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CORRECTION

Volume 6, 1960

Page 584. In equation [2] "$e^{-x/v}$" should read "$e^{-x/v}$".

Volume 7, 1961

Pages 641–655. The author has requested that "brachyosis" be substituted for "brachytosis" throughout this paper.

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VOLUME 7 OCTOBER 1961 NUMBER 5

TORULOPSIS VARTIOVAARAI SP. NOV. A NEW YEAST ISOLATED FROM FINNISH SOIL[1]

AUGUSTO CAPRIOTTI[2]

Abstract

A new species of *Torulopsis* which was isolated from Finnish soil is described. This species is named *Torulopsis vartiovaarai.*

Introduction

In a microbiological study of 32 soil samples from Finland, made in the summer of 1957, 258 strains of yeasts were isolated. These comprised 22 different species; 15 of them already described, whereas the other 7 were considered as new species or varieties. The present paper presents a description of a new species,[3] belonging to the genus *Torulopsis*, which the author proposes to name *Torulopsis vartiovaarai*, in honor of doctor Unto Vartiovaara and other colleagues of the Department of Microbiology, University of Helsinki, Finland.

Materials and Methods

Four strains were isolated from a sample of soil from Viik-Helsinki (Province of Nylandia), Finland. The predominant vegetation was *Lactuca sativa* and the sample was taken at a depth of 2–10 cm. It was gray-colored, sandy-argillous without $CaCO_3$, and had a pH = 5.2.

For the determination of yeast characteristics the technical procedure by Lodder and Kreger-van Rij (3) was applied except for the method concerning the study of sugar assimilation. For these tests, the boiled and washed agar method previously described by Capriotti (1) was utilized.

[1]Manuscript received December 9, 1960.
Contribution from Instituto di Microbiologia Agraria e Tecnica dell' Università di Perugia, Italy.
[2]Present address: Marine Laboratory, University of Miami, Florida.
[3]The writer is indebted to Mrs. N. J. W. Kreger-van Rij (Delft, Holland) for confirming this conclusion.

[The previous number of Can. J. Microbiol. (7, 427–679) was issued September 6, 1961.]

Results

Growth in Malt Extract

After 3 days at 25° C the cells are round with a diameter of 2.5–4 μ or oval (3.5–7 × 3.5–8.5 μ); single, in pairs or groups (Fig. 1). After 1 month at 17° C there is a pellicle and a well-developed ring which is white-colored, and there is rather turbid liquid with good sediment.

Growth on Malt Agar

After 3 days at 25° C the cells are round with a diameter of 2–7.5 μ and oval (2.5–6.5 × 3.5–10 μ); single, in pairs or groups. After 1 month at 17° C the streak culture is white-yellowish, with abundant growth and rather prominent, glistening, waxy, smooth surface and margin. In old cultures some round cells with a diameter of 8–9 μ and sometimes larger, often with an oil drop inside, are found.

Growth on Potato Glucose Agar

After 3 days at 25° C the cells are round and oval (2.5–6 × 3.5–9 μ), single or in pairs. The streak culture is white-yellowish, glistening, and does not develop well. After 1 month at 17° C the streak culture is white-ochraceous, waxy, not well developed, but does have a smooth surface and margin.

Growth on Bean Agar

After 3 days at 25° C the cells are round or slightly oval (3–6.5 × 3.5–8.5 μ); single or in pairs. After 1 month at 17° C the streak culture is white-yellowish, glistening, waxy, and has the characteristic smooth surface and margin.

Growth on Carrot Agar

After 3 days at 25° C the cells are round to slightly oval (2.5–6 × 3–9 μ). After 1 month the streak culture is off-white, glistening, abundant, and smooth.

Must[4] *Gelatine Stab*

After 60 days at 18–20° C there is only a surface colony of white-yellowish color, expanded, and not prominent. No liquefaction is observed.

Giant Colony on Must Gelatine

After 45 days the giant colony appeared rather prominent with a smooth cavity in the center and a furrowed and lobate crown (Fig. 2). There are round cells, long, and narrow cells, which often have one to two oil drops inside.

Sporulation

Spores were not observed.

Pseudomycelium

In slide culture the pseudomycelium is not formed.

Fermentation

Ferments glucose, sucrose (slow), and maltose (weak). Galactose, raffinose, and lactose are not fermented.

Sugar Assimilation

Glucose, maltose, and sucrose are assimilated. Galactose, lactose are not assimilated.

[4]Grape juice.

PLATE I

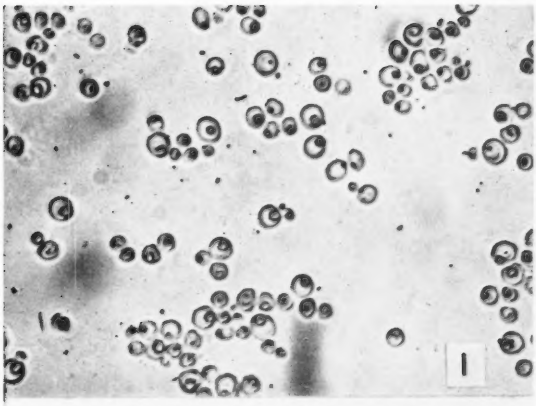

FIG. 1. *Torulopsis vartiovaarai* nov. spec. (×600) (after 3 days on malt extract).
FIG. 2. *Torulopsis vartiovaarai* nov. spec. (giant colony on must-gelatine after 4 weeks).

Assimilation of N-Substances
Ammonium sulphate, asparagine, and potassium nitrate are assimilated.

Growth in Vitamin-free Medium
Positive.

Ethanol as Sole Carbon Source
Growth rather scarce.

Splitting of Arbutin
Positive.

Fermentative Power in Grape-must
Alcohol produced: 0.6–0.8% by volume.

Discussion

The isolated strains are reported to belong in the genus *Torulopsis* because the cells are round to oval, have no spores, form no pseudomycelium, and contain no carotenoid pigments. Lodder and Kreger-van Rij (1952) reported in the genus *Torulopsis* only two species capable of fermentation and assimilation of glucose, maltose, and sucrose: (1) *T. colliculosa* (Hartm.) Sacc. and (2) *T. globosa* (Olson et Hammer) Lodder and Kreger-van Rij. *T. vartiovaarai* differs from these two species in the following ways:

(1) *T. colliculosa* is smaller, ferments and assimilates raffinose, does not assimilate potassium nitrate or ethanol, and does not split arbutin.

(2) *T. globosa* is smaller, weakly ferments glucose, ferments and assimilates raffinose, assimilates ethanol slightly, and does not split arbutin. Since 1952 more than 15 new species or varieties of *Torulopsis* have been described by different authors. Among these only *T. oshoensis* Dietrichson (2) ferments maltose, but it differs from *T. vartiovaarai* in that it does not ferment and assimilate sucrose; assimilates galactose, and does not utilize ethanol, assimilate potassium nitrate, or split arbutin.

Latin Diagnosis

Torulopsis vartiovaarai species nova

In malto trium cellulae globose 2.5–4 μ, vel leniter obovatae 3.5–7 $\times$ 3.5–8.5 μ singulae vel binae. Elapso mense fit degiman, medium parvum nitidum, anulum et album velamen in superficie.

Maltato in agaro cellulae globose 2–7.5 μ et cellulae ovatae 2.5–6.5 $\times$ 3.5–10 μ, singulae vel binae.

Peracto mense 17 C. patina albiflava, copiosa, levigata, glabra, perlucens, aequa, cerea, margine levi.

Pseudomicelium non gignit. Nulla sporificatio observatur.

Glucosus, maltosus (tenuiter), saccharus (leniter) fermentantur. Galactosum, raphinosus, lactosus non fermentatur.

Glucosus, maltosus, saccharus assimilantur. Galactosus, lactosus non assimilantur.

Nitras kalicus assimilatur. Minerali in medio cum alcohole aethilico parumper crescit.

Arbutinum finditur.

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GROWTH OF ENTAMOEBA INVADENS IN ORGANOTYPIC CULTURES OF EMBRYONIC CHICK INTESTINE[1]

E. MEEROVITCH

Abstract

Entamoeba invadens from axenic and monoxenic cultures was inoculated into explants of embryonic chick intestine, which were then cultured in perfusion chambers at 30° C. The growth and metabolic activity of the explants in cultures were evaluated in terms of fibroblastic outgrowth and extent of liquefaction of the plasma clots in which they were embedded. The effects of several media used to fill the perfusion chambers on the survival of the explants were studied. It was found that amoebae developed best in those explants which themselves showed most vitality; this was in turn related to the kind of fluid medium used in the culture. Amoebae in the explants fed on mucous secretion and on dead cells and penetrated into intact tissue without apparent histolytic activity. It is suggested that the living explants provided the amoebae with certain enzymes which the latter were unable to produce at the temperature of incubation. Approximately 40% of all cultures made became positive for amoebae. This is attributed to the fact that not all explants retained the amoebae injected into them, before they were placed in culture.

Introduction

The use of tissue culture in the cultivation of parasitic protozoa including amoebae was recently reviewed by Pipkin (13). Publications not included in this review are those on the growth of an Acanthamoeba in monkey kidney tissue culture (3, 5). Bacteria-free cultivation of *Entamoeba invadens* in a medium containing fresh liver, which does not survive in culture, does not help in understanding the mechanism by which the parasites are able to invade living tissue. Because parasitic amoebae are not intracellular parasites it was hoped that their relationship to the host tissue could best be demonstrated on a whole organ or at least on organized tissue. In order to find this mechanism in living tissue, uncomplicated by the presence of bacteria, attempts were made to grow the amoebae in explants of embryonic chick intestine. Tissue culture methods were used in this work but the technique is more correctly called organ culture.

Materials and Methods

Amoebae

The amoebae used in these experiments were *Entamoeba invadens* (five strains) grown routinely in axenic culture according to the method of Stoll (15) and in monoxenic culture with a penicillin-inhibited *Bacteroides* sp. as the only bacterial associate (14).

In the stock cultures the raw chicken liver extract in Stoll's medium was sometimes replaced by chick embryo extract made either from homogenized 13-day-old embryos (EE_{50} in Tyrode's solution) or by slow extraction in

[1]Manuscript received April 19, 1961.

Contribution from the Institute of Parasitology, McGill University, Macdonald College P.O., Que., Canada, with financial assistance from the National Research Council of Canada and from the National Academy of Sciences – National Research Council (United States) in the form of a Donner Post-Doctoral Fellowship.

buffered saline at pH 7 in the cold. Most of our strains of *E. invadens* produce better cultures in media enriched with embryo extract than with raw liver extract.

Intestinal Explants

These were derived from the duodenum of 13- to 15-day-old White Leghorn chick embryos. The lengths of intestine taken were those between the crop and the gall bladder. In a successful operation the uninterrupted length of intestine removed was about 2 cm.

At the 13- to 15-day stage the embryonic chick intestinal mucosa has the characteristic "zigzag" pattern of previllous ridges (2). It was hoped that the previllous ridges would differentiate into villi during the period of growth *in vitro*, but this was not achieved.

Culture Media

Chicken Plasma

Chickens were starved overnight and bled by cardiac puncture. Blood was collected in chilled, paraffin-coated 40-ml screw-cap tubes, each containing 6 mg of heparin, and centrifuged in a refrigerated centrifuge at 3000 r.p.m. for 30 minutes. The heparinized plasma was kept in the refrigerator in paraffin-coated screw-cap tubes.

Chick Embryo Extract

Thirteen-day-old embryos, with their eyes, beaks, and feet removed, were washed in several changes of Tyrode's solution and homogenized in a Tenbroek tissue homogenizer. A volume of Tyrode's solution equal to twice the volume of the embryo homogenate was added and the mixture placed in the cold for about 30 minutes. It was then centrifuged in a refrigerated centrifuge at 2500 r.p.m. for 5 minutes, the supernatant pipetted off into screw-cap tubes, quick-frozen in dry ice, and stored in a deep-freeze cabinet. The customary sterile precautions were observed throughout.

Fluid Media

Several modifications of media used to fill the chambers of the perfusion slides, described below, were tried during the course of this work. The purpose was to devise a medium which would fulfill the requirements of both the constituents of the culture, that is the intestinal explant and the amoebae.

The explants themselves do not require a fluid medium as they are able to grow in chicken plasma – embryo extract clots. This technique was first described by Fell and Robison (4). Because the purpose was to enable the amoebae to multiply as well, the fluid medium had to be such as not only to inhibit the growth and metabolism of the explants, but to favor the multiplication of the amoebae.

Tissue culture Medium 199 (Difco, dried) was used as the base for the various modifications. When the fluid medium contained additional ingredients in solution, Medium 199 was prepared in double strength so that when diluted with the other ingredients dissolved in water the final concentrations of its numerous components were of the normal value.

The serum used throughout was Bacto Beef Blood Serum dissolved according to instructions and sterilized by Seitz filtration. Penicillin and streptomycin

were added to the complete medium in final concentrations of 100 i.u./ml and 100 μg/ml, respectively.

Additional ingredients used in some experiments will be described below.

Methods and Apparatus

The lengths of embryonic intestine were washed free of blood in several changes of warm Tyrode's solution. Adhering bits of mesentery, blood vessels, and glandular tissue were dissected away from the intestines.

Cultures of *E. invadens* were concentrated by centrifugation (1000 to 1500 r.p.m. for 5 to 10 minutes); the amoebae were washed two or three times with sterile saline and then resuspended in a few drops of the culture medium to be used in the experiment. The saline used in washing the amoebae from the monoxenic cultures contained 500 to 1000 i.u. penicillin/ml in order to kill any *Bacteroides* not completely inhibited.

Cysts were never present in the axenic cultures, but were sometimes present in the monoxenic ones.

The washed amoebae were taken up into a tuberculin syringe with a gauge-27 hypodermic needle or into an "Agla" micrometer syringe (Burroughs Wellcome and Co., London). The volume of the amoebic suspension was seldom greater than 0.05 ml.

The lengths of intestine were placed on sterile 7.5×2.5 cm microscope slides which were in turn placed on top of 7.5×4.0 cm slides and cemented to them by running a drop of water or saline between them. The slides with the intestines upon them were placed on the stage of a binocular dissecting microscope which was protected from falling dust by a specially fitted "Perspex" hood, open at both sides to allow for manipulation.

The intestines were injected into the lumen with the amoebic suspension, the operation being observed through the microscope. When a tuberculin syringe was used, the operation required two persons, one to push the plunger and the other to hold the intestine with fine forceps and to guide the hypodermic needle. The use of the "Agla" micrometer syringe mounted in a clamp stand enabled one person to perform the inoculation. No attempts were made to count the number of amoebae injected. Care was taken not to rupture the wall of the intestine and to fill them with amoebic suspension without air bubbles. The manipulation of the intestine nearly always produced intense muscular contractions which often made the inoculation quite difficult.

After several lengths of intestine were inoculated they were set aside in a sterile petri dish for a few minutes to allow the amoebae to settle on the mucosa. Then the lengths of intestine were cut into shorter lengths of about 2 mm with fine cataract knives and were washed again in the culture medium to remove any amoebae which might have spilled out or happened to be on the exterior. These 2-mm-long explants were embedded in chicken plasma–embryo clots on 22-mm² cover slips (one drop of each, making a circular clot about 6 to 8 mm in diameter). After the clots were hardened, which usually took not less than 15 to 20 minutes, the cover slips were inverted over the circular chambers of "Perspex" perfusion slides and were cemented to them with melted paraffin. The perfusion chambers (Fig. 1) were made by cementing together two pieces of "Perspex", one of which had the central culture chamber and

the two side holes drilled through it. The dimensions of the chambers are given in the illustration. When the paraffin became hardened the chambers were filled through one of the side holes with the culture medium by means of a fine Pasteur pipette, care being taken not to leave air bubbles in the chambers. The side holes were sealed with paraffin and the cultures were incubated in a horizontal position at 30° C.

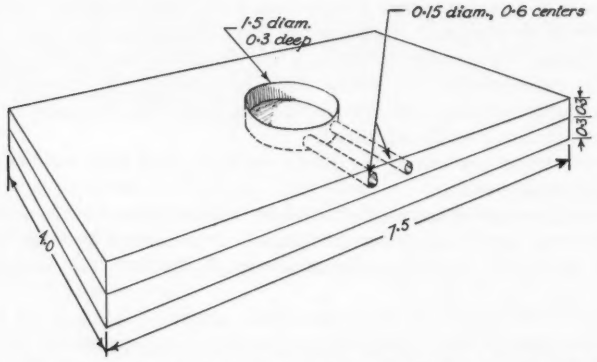

FIG. 1. "Perspex" perfusion chamber. (All dimensions in centimeters.)

Methods of Observation

The cultures were routinely examined under a phase contrast microscope. The "Perspex" chambers, filled with the medium and sealed with glass cover slips, provide a very satisfactory optical system for viewing by this method. However, due to the thickness of the chambers, only the 10×/0.25 mm objective could be used.

The amount of fibroblastic outgrowth and the extent of liquefaction of the plasma clot were evaluated on an arbitrary scale which ranged from (+), denoting very few outgrowing cells or a very small area of liquefaction, through 1, 2, and 3, which marked progressively larger areas of outgrowth and liquefaction. The explant shown in Fig. 2 was evaluated 2 for outgrowth and 3 for liquefaction.

For histological study the explants were dissected out of the plasma clots, fixed in Zenker's formalin or in buffered neutral formol-saline, and the sections were stained with haematoxylin–eosin, haematoxylin – naphthol green B – aniline eosin, or haematoxylin – alcian blue – diphenyl fast red.

Sterility Checks

Culture media, amoebic inocula, and contents of culture chambers upon termination of cultures were routinely tested for bacterial or fungal contamination, in Brewer's thioglycollate medium. Contaminated cultures were discarded and not included in the final analysis of results.

Amoebae from monoxenic cultures, after being washed in a penicillin solution, were generally free of viable bacteria.

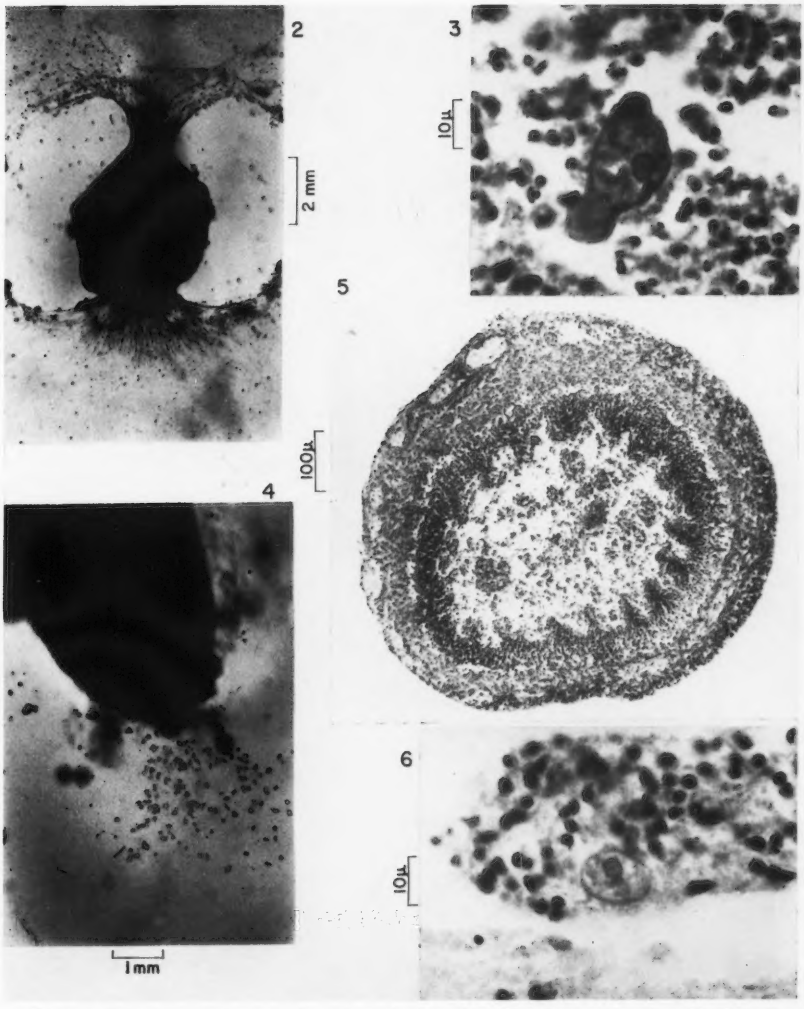

FIG. 2. General appearance of an intestinal explant in culture, showing fibroblastic outgrowth and pools of liquefied plasma.

FIG. 3. An amoeba in an area of tissue debris, showing ingested nuclei and cell fragments.

FIG. 4. Part of intestinal explant, showing amoebae concentrated near one of the cut ends.

FIG. 5. Cross section through an intestinal explant from culture, showing necrosis of the mucosa.

FIG. 6. An amoeba embedded within a villus of an intestinal explant in culture.

Observations and Results

In the perfusion slide cultures fibroblastic outgrowths from the explants began to appear about 24 hours after explantation. At first they appeared at the two cut ends of the explants but later they were sometimes seen to originate also along the intact slides. After 2 or 3 days the fibroblastic outgrowths formed themselves into cords and sheets and became arranged in an arc of a circle at either of the cut ends. Very often these fibroblastic outgrowths arranged themselves in a pattern reminiscent of that formed by magnetic lines of force around a bar magnet (Fig. 2).

The explants continued to undergo peristaltic movements during the first day or two; such peristaltic activity in explants of embryonic intestine was studied by Bisceglie (1).

At the temperature of incubation (30° C) the whole pattern of growth was considerably retarded from that at 37° to 38° C. This was considered an advantage because the cultures could be maintained for as long as 10 days. A temperature of 30° C is approximately the highest at which *E. invadens* is able to grow and multiply without a gradual adaptation to higher temperatures, and consequently it was chosen as a reasonable compromise between the temperature requirements of the explants and the amoebae. Previous experience with cultivation of *E. invadens* in living chick embryos had shown that this temperature could be used (7).

Plasma clots in which the explants were embedded became liquefied in areas adjacent to the two cut ends, so that fibroblastic outgrowths surrounded a pool of liquefied plasma (Fig. 2). There was an almost continuous extrusion of a granular and mucous material from within the explants during the first few days of culture; this extruded material, which is believed to be dead tissue, settled on the bottom of the perfusion chambers.

The amoebae began to make their appearance in the liquefied plasma pools usually not earlier than 3 days after initiation of the cultures. This sequence of events was a fortunate coincidence. The amoebae, even had they multiplied sufficiently within the explants so as to spill out of them, could not do so before the plasma was liquefied. Consequently, the emergence of the amoebae out of the explants, after plasma liquefaction had taken place, enabled them to move and to multiply outside of the explants. Control experiments have shown that amoebae are killed very soon after being embedded in plasma clots. It was frequently seen that whenever an amoeba happened to be on the exterior of an explant after the latter was embedded in the plasma clot, it invariably died and disintegrated very soon afterwards. It is, therefore, safe to state that the amoebae which were eventually observed in the perfusion chambers came originally from within the explants.

Effect of the Fluid Media on the Explants

As stated above, the growth of the explants was evaluated in terms of two factors: (1) the extent of the fibroblastic outgrowth and (2) the extent of the liquefaction of the plasma clot. Both these manifestations are sound indicators of the viability of the explants. However, it was seen that a large area of liquefaction, unaccompanied by a heavy fibroblastic outgrowth, meant that culture conditions were not very favorable. A heavy fibroblastic outgrowth

with little or no liquefaction probably meant that the outgrowth originated not from the intestinal explant itself but from bits of connective tissue adhering to it. In such cases amoebae were not seen in cultures.

As already mentioned, the explants do not require a liquid phase of the medium in order to grow, but it was necessary for the requirements of the amoebae. It was seen that certain media were detrimental to the survival and the physiological activity of the explants. It was concluded, therefore, that the fault of such media lay not in their lack of certain necessary ingredients but in the inhibitory or antagonistic effects of some of their constituents. It will be shown below that growth and multiplication of the amoebae depended on the vital state of the explants; consequently, these media, which were not inhibitory for the explants, were the same ones which were favorable for the amoebae.

Fluid media used contained phenol red and they were always adjusted to pH 7 with sodium bicarbonate. The acidity of the cultures increased during the period of incubation. In those cases in which the original medium was replaced by fresh medium (by means of pipetting through one of the side holes), the shift of the pH to the acid side was repeated.

Table I summarizes the data pertaining to 203 cultures. Medium 199 supplemented with 10% bovine serum produced outgrowth and liquefaction to the mark of 3, while when it was supplemented with 10% to 25% chick embryo extract, liquefaction and outgrowth did not exceed the mark of 1. Addition of both the serum and the embryo extract produced outgrowth and liquefaction intermediate between that produced by either of the two supplements used separately.

Stoll's medium, which is used for the axenic cultivation of *E. invadens*, contains 25% by volume of a 2% solution of Trypticase (B.B.L.) and about 28% by volume of a 0.5% autoclaved and Seitz-filtered solution of mucin. These two supplements were added in approximately similar concentrations to Medium 199, in addition to serum or embryo extract, in order to test the ability of this enriched medium to support the outgrowth of the amoebae, without inhibiting that of the explants. The final concentration of mucin in the complete medium was 0.11% and that of Trypticase 0.44%.

As shown in Table I, addition of mucin and Trypticase improved somewhat the adverse effect of the embryo extract (the value being 2 for both the outgrowth and liquefaction). Nevertheless, the same combination of ingredients, but with serum instead of embryo extract, produced outgrowth and liquefaction to the mark of 3. Medium 199 with mucin, Trypticase, embryo extract, and serum produced outgrowth and liquefaction only to the mark of (+). Thus, the deleterious effect of embryo extract on the growth of intestinal explants was still retained.

Addition of 0.1% lactalbumin hydrolyzate to Medium 199 enriched with mucin, Trypticase, and serum did not have any effect on outgrowth and liquefaction.

The effect of the addition of pig duodenal mucosa acetone powder extract to Medium 199 with either embryo extract or serum was investigated. The acetone powder was suspended in water to make a known g/ml concentration. Not all of it dissolved, but the resulting solution was arbitrarily assigned

TABLE I

Growth of chick embryo intestinal explants and *E. invadens* in Medium 199 supplemented with additional ingredients
(Concentration expressed as percentage of total medium)

Mucin	Trypticase	Lactalbumin hydrolyzate	Mucosal extract	Embryo extract	Bovine serum	Mean*		No. of cultures	No. of cultures positive for amoebae	Percentage of cultures positive for amoebae
						Growth	Liquefaction			
					10	3	3	10	8	80
				10–25		1	1	20	1	5
				12.5	9	2	1	6	0	0
0.11	0.44			10–12.5		2	2	65	17	26
0.11	0.44				10	2–3	2–3	49	35	71
0.11	0.44	0.1			10	2	2	6	3	50
0.11	0.44			10	10	(+)	(+)	3	3	100
			0.12	10		1	1	2	0	10
			0.25	10		1	1	2	0	
			0.5	10		1	1	6	1	
			0.12		10	2	2	2	1	20
			0.25		10	2	2	16	3	
			0.5		10	3	3	2	0	
	0.25		0.25		10	3	3	14	7	50
								203	79	39

*See section "Methods of Observation" for evaluation of growth and liquefaction.

the concentration of the original suspension. The mixture was centrifuged and passed through a Seitz filter, and the resulting clear solution then used as the base to make the final medium. The results, as seen in Table I, showed that although no marked difference was observed from cultures in which no mucosal extract was used, the explants in the medium containing serum did better than those in the medium containing embryo extract. Thus, mucosal extract did not improve the quality of the medium containing embryo extract. Moreover, the addition of the mucosal extract produced a very heavy precipitate around the explants.

Medium 199 enriched with Trypticase (0.25%), duodenal mucosa extract (0.25%), and serum (10%) produced very good outgrowth and liquefaction (Table I).

Medium 199, not supplemented by any additional ingredients, did not produce growth or liquefaction beyond the mark of 1.

Effect of the Fluid Media without Intestinal Explants on Amoebae

Several control experiments were set up at 25° and 30° C to determine whether the fluid media used in the perfusion chamber cultures would support the growth of *E. invadens* in the absence of any tissue.

It was found that amoebae did not survive or multiply in any of these media, either in test tube cultures under a petroleum jelly seal (as in routine axenic cultures), or in the perfusion chamber cultures. In addition to this, in every experiment with the perfusion chambers, a few of these were inoculated with amoebae but did not contain an intestinal explant. The medium in each case was identical with the one used in the experimental cultures. In no case did amoebae survive in the absence of an intestinal explant.

In a separate experiment, axenic *E. invadens* was inoculated into a series of tubes containing fragments of chick embryo intestine in three types of media: (1) Medium 199, mucin, Trypticase, and serum; (2) Medium 199, mucin, and serum; (3) Medium 199, Trypticase, and serum. The poorest growth of amoebae occurred in medium 2, while those in media 1 and 3 were almost identical. There was no growth of the intestinal fragments and they soon became necrotic and were eaten by the amoebae.

These control experiments and cultures proved that the presence of intestinal explants was essential for the multiplication of amoebae, even in the enriched and complex media which were used.

Appearance and Behavior of Amoebae in Cultures and their Relationship to the Living Substrate

Amoebae which emerged from within the explants after the plasma clots became liquefied, and subsequently multiplied in the surrounding medium, were active and often contained fragments of cells and debris (Fig. 3). Soon after their emergence, the amoebae aggregated near the cut ends of the explants (Fig. 4), but eventually they became dispersed throughout the chamber.

When the fibroblastic outgrowths produced cords of cells, the amoebae were mostly seen to aggregate on or in close proximity to these cords. In older cultures, which contained amoebae, the cords eventually became broken down to a series of isolated islands which were surrounded by amoebae. In control cultures, which did not contain amoebae, the fibroblastic cords

persisted longer, and when the cells died the cords disintegrated without breaking up into islands. Amoebae were also seen to aggregate in the proximity of the extruded granular material which settled on the bottom of the chambers.

As seen in Table I, those cultures in which the explants produced most outgrowth were the ones in which *E. invadens* developed best. Notably very good results were obtained with Medium 199, mucin, Trypticase, and serum (71% of these cultures became positive for amoebae).

The average percentage of all cultures in the different media which became positive for amoebae was approximately 40. The approximate percentage of all cultures in which axenic *E. invadens* was used and which became positive for amoebae was 40, and that in which monoxenic *E. invadens* was used was also 40.

No differences were noted in the response between the different strains of *E. invadens*.

It may thus be said that this technique is efficient only to the extent of approximately 40%.

Microscopical Examination of Cross Sections of Intestinal Explants from Cultures

Intestinal explants taken from cultures, fixed, sectioned, and stained, in the majority of cases showed rather severe signs of necrosis. Monesi (11) also found necrosis occurring in the center of chick embryo intestinal explants grown in plasma clots, as early as 4 days after explantation. The degree of necrosis in our material increased the more the longer the culture was kept. As stated earlier, necrotic tissue and debris were extruded by the explants during the first few days of culture. Figure 5 shows a cross section of an explant fixed 6 days after explantation, revealing necrosis of the mucosa. Controls, that is uninjected explants, underwent necrosis in the same manner as the injected explants.

Amoebae were seen in the lumina of the explants, as well as within the tissues. In explants which were fixed early, the amoebae were seen only in the superficial tissues (mucosa and submucosa), while in those which were fixed later, after the amoebae had already multiplied in the medium outside, the latter were often seen in the deeper tissues, indicating that invasion may have occurred from the exterior.

No pathological changes which might be described as localized lesions or ulcers were seen. Amoebae in tissues were usually seen singly or in small groups. No lysis of the surrounding tissue was observed, neither were there signs of tissue damage that could be attributed to the penetration by the parasites. Figure 6 shows an amoeba embedded well within a villus (or a previllous ridge). It will be seen that the mucosal tissue is in a fairly good state of preservation and that there is no sign of lysis or of mechanical damage.

Sections of explants from cultures stained with alcian blue showed the typical histochemical color reaction for mucus. Mucosa and the amoebae in the lumen or within the mucosa stained deep blue, while those which had penetrated deeper into the intestinal wall did not. This suggests that while in the lumen, the amoebae fed on mucus. This observation parallels an earlier one, based on *in vitro* work and *in vivo* in snakes (8, 9).

Discussion

These experiments have shown that the multiplication of *E. invadens*, injected into explants of chick embryo intestines, was dependent on the vital state of the explants. It is known that *E. invadens* can be grown axenically in a medium consisting of dilute serum and a fragment of tissue such as liver (6, 10). The tissue in such cultures is certainly not alive and the amoebae merely utilize the dead cells as food. *Entamoeba invadens* will grow axenically at temperatures up to 26° C, and only with difficulty at 30° C. As our intestinal explant – amoebae cultures were incubated at 30° C and luxuriant growth of amoebae obtained, perhaps the metabolizing explants provided a source of enzymes which the amoebae themselves were unable to elaborate at this high temperature.

This hypothesis could explain why amoebae failed to develop in those cultures in which the explants did not survive. It must be remembered that the control experiment, in which *E. invadens* was grown successfully in test tubes in the tissue culture media with fragments of embryonic intestine, was conducted at 25° C.

The lack of tissue lysis around the amoebae in the intestinal tissue suggests that tissue damage in amoebiasis is due to a large extent to bacterial invasion and not to a histolytic activity of the amoebae. Work with axenic *E. histolytica* and germ-free animals showed that no tissue invasion by the amoebae can take place in the absence of bacteria (12).

The reason why only about 40% of all cultures became positive for amoebae probably lies in the technical imperfection of the method and presumably about 60% of the explants failed to retain the amoebae inoculated into them, after they were cut into 2-mm lengths.

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ISOLATION OF AND STUDIES ON BACTERIOPHAGE ACTIVE AGAINST MYCOBACTERIA[1]

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Abstract

More than forty bacteriophages lytic for saprophytic mycobacterial strains have been isolated from soil. Seven were isolated without enrichment, one following enrichment with ATCC No. 607 and *Mycobacterium phlei*, and the remainder following enrichment with ATCC No. 607 only. Enrichment with ATCC No. 607 followed by *M. phlei* resulted in isolation of a phage which lysed specifically the *M. phlei* strain. The lytic patterns of 22 of these phages have been determined on 31 bacterial strains. No lysis of pathogenic mycobacteria by the phages isolated from dung soil has been observed.

Acid-fast bacteria isolated from the soil from which the phages were isolated showed varying susceptibilities to the phages.

Antiserum produced in rabbits against one of the phage strains inactivated 97% of the homologous phage at 1:100 dilution in 30 minutes. There was no effect on the other phage strains.

Electron micrographs showed marked differences in the particles of two of the phage strains. Tests of susceptibility to ultraviolet irradiation and to heat indicated wide variations in six phages.

Introduction

Since the isolation, by Gardner and Weiser (5), of bacteriophage active on mycobacteria, several attempts have been made to utilize phage-typing as a method of differentiating and classifying the acid-fast bacteria (4, 7, 8, 10). Although numerous strains of phage lytic for mycobacteria have been isolated and tested, only relatively few have been found to lyse the pathogenic and atypical strains. This is not too surprising since most phages have been isolated from soil where the presence of saprophytic mycobacteria would be expected but where pathogenic human strains would not as likely be found. Another factor that has retarded the development of phage-typing in the mycobacteria is the fact that few, if any, phages specific for one strain of bacteria have been found.

In the present study more than forty strains of phage have been isolated. Only 22 have been studied with sufficient detail to justify even preliminary conclusions. Two strains of phage have been adapted each to a strain of mycobacteria for which it was not originally lytic. Tests indicate that the adapted phages are specific, each lysing one host only.

Materials and Methods

Bacterial Strains

The strains of bacteria used in the isolation and testing of the phages were common laboratory stock strains, variants of these strains, and strains iso-

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lated from soil. The principal strain used in isolation and for the phage studies was the American Type Culture Collection No. 607 of *Mycobacterium* sp. The 607L (R1) strain is 607 made lysogenic with the temperate R1 phage of Bowman and Redmond (3), and the DSX_1 and DSX_{17} strains are bacilli from the 607 strain made lysogenic with DS phages of unknown affinities. The DSX_1 and DSX_{17} strains were isolated from a plate before the phages had been isolated and given numerical designations. The seven C-strains are acid-fast bacteria isolated from soil samples from the same location as the soil from which the DS phages were isolated. The 28W and 28Y are white and yellow variants of *M. butyricum* (R1), a naturally lysogenic strain.

Isolation of Phage

Soil was obtained from two sources, a vegetable garden and a stockyard (dung soil). For direct isolation, samples of garden soil and stockyard soil, respectively, were mixed with nutrient broth (Difco) and the mixtures were incubated at room temperature for 1 hour with several agitations. The supernatant was then removed and passed through an ultrafine sintered glass filter. Samples of the filtrates were tested on several saprophytic and pathogenic strains of mycobacteria for the presence of phage.

The enrichment procedure was adapted, with slight modification, from the method used by Hnatko (6). Soil samples of approximately 40 g each in 250-ml Erlenmeyer flasks were enriched every fifth day by the addition of 5–7 ml suspensions of a single saprophytic strain of mycobacteria. Each suspension consisted of a 24-hour aerated broth culture (20 ml) centrifuged and resuspended in 5 ml of nutrient broth. The enrichments were carried out for 4 weeks at the end of which time filtrates of each flask were tested for phage on several strains of mycobacteria. Pure strains of phage were obtained from isolated plaques.

Media

Nutrient broth (Difco) was used for the propagation of the saprophytic strains employed in the enrichments and for testing. Tests for phage were made by plating samples of the filtrates mixed with saprophytic acid-fast bacilli on nutrient agar using the soft agar method as modified by Bowman (2). When pathogenic and unclassified strains were used platings were made on a modified Dubos oleic acid albumin agar. The Dubos agar was made up as follows: KH_2PO_4, 1.0 g; Na_2HPO_4 (anhyd.), 2.2 g; asparagine, 2.0 g; casein hydrolyzate, 2.0 g; $MgSO_4$, 0.01 g; ferric ammonium citrate, 0.05 g; $CaCl_2$, 0.01 g; $ZnSO_4$, 0.0001 g; $CuSO_4$, 0.0001 g; agar, 11.0 g; glucose, 10.0 g; oleic acid – albumin complex, 100 ml per liter. The glucose and the oleic acid – albumin complex were sterilized separately and added after autoclaving and cooling the agar base to 48° C.

Antiserum

Antiserum was prepared for the DS_1 phage by injecting intravenously into rabbits 5 to 7 ml of a phage suspension containing 1 to 2 $\times 10^{11}$ plaque formers per ml every other day until 15 injections were made. After a period of 6 weeks the animals were bled by heart puncture. The blood was allowed to clot overnight in the refrigerator, centrifuged, and the serum removed and stored at 4° C.

Ultraviolet Irradiation

The susceptibility to ultraviolet irradiation was determined on several strains of the phage with periods of irradiation of 1, 2, and 4 minutes. Irradiation was carried out with the surface of the suspensions 17.5 in. from the center of the light. A 17-watt General Electric slimline germicidal lamp was used. The phage was diluted to contain 5×10^4 particles per ml and was suspended in phosphate buffer (pH 7.0) containing 1% bovine albumin. Eight milliliters of the suspension was placed in a petri dish and agitated during irradiation. Aliquots were removed at intervals and plated on ATCC No. 607. All manipulations were carried out in dim light to avoid photoreactivation.

Electron Microscopy

A Spinco model L centrifuge was used in the preparation of the phage samples. The suspension was centrifuged at 5,000 rpm for 30 minutes, the supernatant was pipetted off and then centrifuged at 20,000 rpm for 30 minutes. The supernatant was discarded and the sedimented phage was resuspended in nutrient broth. This process was repeated one or two times. The concentrated specimens were deposited on collodion or carbon covered grids and allowed to air-dry partially. This method was previously described by Backus and Williams (1) and used by Bowman (2). The specimens were examined with an RCA, EMU-2 electron microscope.

Results

Isolation and Host Ranges

Five phage strains were isolated from garden soil without enrichment. These have been designated GS_1 to GS_5. From the dung soil approximately 40 phages have been isolated and these have been designated as DS phages with consecutive numbers beginning with 1. Of these, DS_1 and DS_{25} were found in dung soil samples prior to enrichment; the remainder were isolated following enrichment. The DS_2 phage was isolated after enrichment with ATCC No. 607 for 3 weeks followed by enrichment with *M. phlei* for an additional 3 weeks.

A partial list of hosts and the host ranges of 22 of the GS and DS phages is to be found in Table I. It is apparent that the phages have widely different affinities for most of the strains of bacteria tested. Very little relationship is shown except in the five GS phages and even among these there are varying patterns of lysis. The uniform action on the 607 strain is a reflection of the use of this strain for isolation of the phages.

The results indicate a close relationship of the *M. ranae* and the ATCC No. 607 strains. The C strains are resistant to lysis by the GS phages but show variable susceptibilities to phages of the DS group. None of the phages lysed *M. phlei* with the exception of DS_2; and the DS_2 phage lysed only the *M. phlei* strain of bacilli.

Plaque Formation

The plaques produced by the different phages of this group vary considerably in size from small pin-point plaques to large 8–10 mm plaques. Some are relatively clear while others are turbid. Eight plaque types are shown in

TABLE I

Action of DS and GS bacteriophages on various strains of mycobacteria

Bacilli	Bacteriophage																				
	GS_1	GS_2	GS_3	GS_4	GS_5	DS_1	DS_2	DS_3	DS_4	DS_5	DS_7	DS_8	DS_9	DS_{10}	DS_{11}	DS_{12}	DS_{15}	DS_{16}	DS_{17}	DS_{18}	DS_{19}	DS_{20}
ATCC No. 607	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
607L (R1)	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+						
Ranae	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Butyricum (R1)	+	−	+	−	−	−	−	−	−	−	−	−	−	−	+	+						
Phlei	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Fortuitum 280	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−						
Fortuitum 290	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−						
28 W	+	+	+	+	+	+	−	−	−	−	+	+	−	−	−	−						
28 Y	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−						
DSX_1	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	+	+	+			+
DSX_{17}	+	−	−	+	+	+	−	+	−	−	−	−	−	−	−	−						
Smegmatis	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Butyricum 11314	+	+	−	+	+	+	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−
Butyricum 280	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Butyricum 357, 358, 359, 360	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Butyricum 362	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Butyricum 1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
Butyricum 2	+	+	+	+	+	+	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−
Butyricum 3	+	+	−	−	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	+	−	+
Butyricum 4	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Buytricum 20	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	−
C-1	−	−	−	−	−	+	−	−	−	−	+	+	−	−	−	−	+	+	+	+	+	+
C-2	−	−	−	−	−	+	−	−	−	+	+	+	−	−	−	+	−	+	+	+	−	+
C-3	−	−	−	−	−	+	−	−	−	+	+	−	−	−	−	−	−	−	+	+	−	+
C-4	−	−	−	−	−	+	−	−	−	+	+	+	+	−	−	+	+	+	+	+	−	−
C-5	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	+	+	+	−	−
C-8	−	−	−	−	−	+	−	−	−	+	+	+	+	−	−	−	+	+	+	+	+	+
C-10	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	+

Note: + = plaques formed; − = no plaques formed.

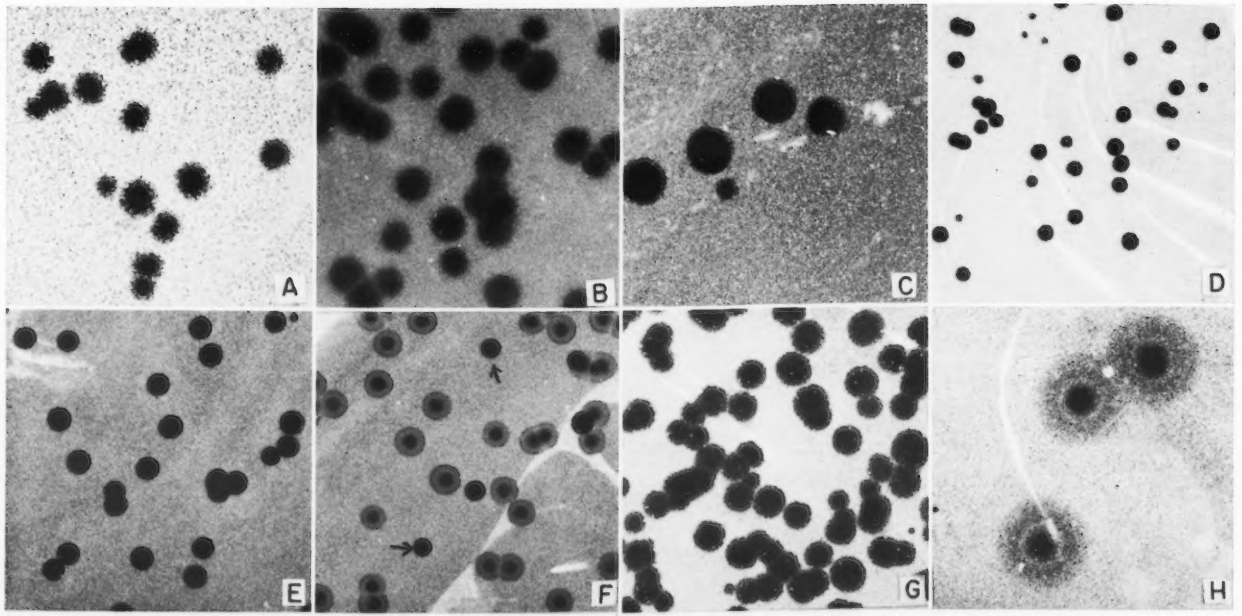

FIG. 1. Plaques of DS and GS phages when plated on mycobacteria using the soft agar layering technique. A, phage DS_2 on *M. phlei*; B, phage DS_3; C, phage DS_4; D, phage DS_8; E, phage DS_{11}; F, phage DS_{12} (DS_{12A} indicated by arrows); G, phage GS_2; H, phage GS_4. B to H, phage plated on ATCC No. 607. All pictures made to same scale.

PLATE II

FIG. 2. Electron micrograph of DS_1 phage (magnification 82,000×).

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Fig. 1. Following single plaque isolations several of the DS phages have given rise to mutants. Mottled plaques have been found in several cases, these generally giving rise to one or more variant-type plaques the phages of most of which are stable. The three phages DS_3, DS_4, and DS_5 together with the parent mottled type result when a single mottled plaque is sampled and the phage is plated on ATCC No. 607 or on *M. rànae*. Phage DS_{12} gives rise to a smaller, clearer variant type DS_{12A} indicated by arrows in Fig. 1 (F). Likewise, surrounding the DS_{15} plaques appear small pin-point plaques. The phage isolated from these small plaques remains true to type, and has been designated DS_{15A}.

Phage Studies

Electron micrograph studies have been made on the DS_1 and GS_2 phages. The DS_1 phage is shown in Fig. 2. The head appears spherical and measures approximately 100 mμ in diameter. The tail is short, being slightly less than 100 mμ in length. The GS_2 phage is only about half as large as the DS_1 phage but has a much longer tail.

Six phages were selected for tests of susceptibilities to ultraviolet irradiation and to heat. In Fig. 3 is shown the results of irradiating this group of phages for 60, 120, and 240 seconds. The results show a separation of the six phages into two groups with different susceptibilities, one group consisting of four strains, the other of two strains. Only a very few plaques were observed

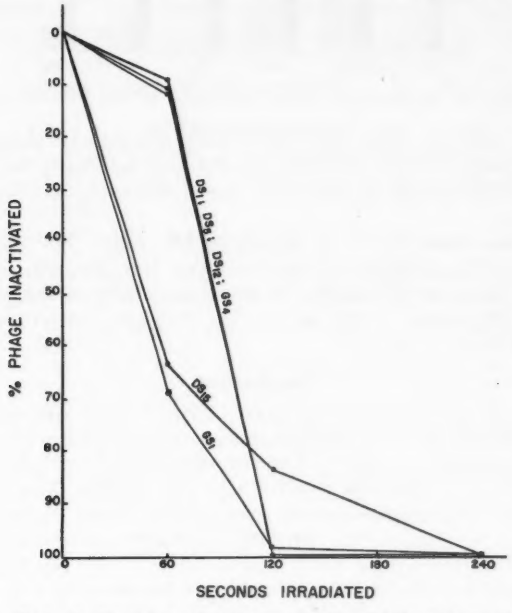

FIG. 3. The effect of ultraviolet irradiation on the activity of four DS and two GS phages.

following irradiation for 4 minutes. This is in contrast to the DS_{6A} phage isolated by Redmond and Cater (9), only 90% of which is inactivated by 10-minute irradiation.

The same six phages were tested for the effect of heating in a water bath at 37° C for 30 minutes, at 56° C for 30 and 60 minutes, and at 70°C for 10 and 30 minutes. The results of the tests at 56° C are shown in Fig. 4. Heating for 30 minutes inactivates 85 to 90% of the phage of four strains (DS_1, DS_{15}, GS_1, and GS_4), but has little or no effect on the other two (DS_5 and DS_{12}). These two strains show marked resistance to 56° C heating even for 60

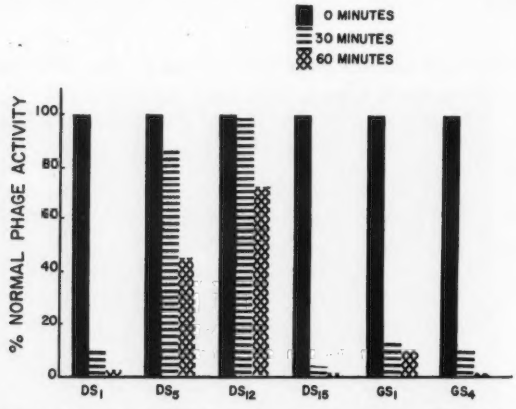

FIG. 4. The effect of heating at 56° C on the activity of four DS and two GS phages.

minutes. Two strains, DS_{15} and GS_4, were completely inactivated in 60 minutes. Very little, if any, effect was observed following heating at 37° C for 30 minutes. No phage activity was noted following heating at 70° C for 10 minutes.

Antiserum has been produced for phage DS_1 only. The phage was incubated with a 1:100 dilution of the antiserum for 10-, 20-, and 30-minute periods, then plated on 607 bacilli. In 30 minutes 97% of the phage is inactivated by a 1:100 dilution of the antiserum. The DS_1 antiserum had no effect on the other DS or GS phages.

Discussion

The phage strains which have been isolated from soil samples raise a variety of problems, one of which is concerned with the origin and relationships of the various phage strains. The variants which have appeared following isolation indicate an unstable condition of the phages. This condition conceivably may be responsible for the many types found in the soil. There is little possibility of defining the variables of such a complex medium as the soil, expecially that obtained from a stockyard. It has been pointed out by Redmond and Cater (9) that many acid-fast bacteria may be destroyed by agents in the soil with possible liberation of deoxyribonucleic acid. Though not affecting the free phage in the soil, deoxyribonucleic acid may very likely

penetrate into a bacterium that has been infected by a phage particle and in this manner modify the offspring of the phage. It is obvious that some phage strains are more susceptible to the influence of external agents than are others. Extensive experimentation will be necessary in order to determine what factors are responsible for the plastic hereditary conditions which have been observed in these phages.

The DS_2 phage appears to be an example of adaptation to a host not previously attacked. Another example of possible adaptation is the DS_{6A} phage active on pathogenic human tubercle bacilli (9).

Information concerning the origin and adaptability of these and other phages could aid in the isolation of phages with specific activity on other strains. The need for phages of this type is particularly great among the groups of the 'unclassified' mycobacteria. At present there is no means of determining the magnitude of the differences that undoubtedly exist among the numerous mycobacterial strains that have been isolated from tuberculous patients. Although the DS_2 phage *per se* has little practical significance the method used in adapting and isolating it could lead to the isolation of phages specific for more important mycobacteria, such as the unclassified groups, and to a better understanding of the factors involved in adaptation of phage. As a result of the present studies, it is evident that highly specific phages can be isolated, since phage specific for *M. phlei* has already been obtained. As reported elsewhere (9) this line of attack has been used in the successful isolation of phage specific for *M. tuberculosis* varieties *hominis* and *bovis*. Should it be found possible to adapt phage to the various strains and groups of the unclassified mycobacteria a means of typing and identifying these important strains would become available.

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INDUCTION AND CHARACTERISTICS OF STAPHYLOCOCCAL L FORMS[1]

L. H. MATTMAN, L. H. TUNSTALL, AND H. W. ROSSMOORE

Abstract

Staphylococcal L forms revealed characteristics not observed previously. The ability to produce coagulase was retained by the variant. In the presence of blood, many L forms were found within red cells, comparable with the intraerythrocytic stages reported for streptococci. Agar lysis was a unique characteristic of the mature L form.

Inhibitors were not required to initiate the L stage and the variant and classical form grew commensally on enriched media. As nutrition was simplified, the L stage was supported, whereas the classical form did not reproduce. A small inoculum resulted in exclusive growth of L forms even in media containing special growth factors. The relative ease with which the L forms are produced experimentally suggests that this phase of growth may occur *in vivo*.

Introduction

World-wide studies now suggest that bizarre filterable growth in the L form may be an inherent property of all bacterial genera. Even more intriguing is the demonstration of spontaneous L-type growth in nature (3) or *in vivo* (1, 5, 12, 21, 34), and the finding that L and classical forms can develop commensally (7, 21). Exclusive growth of the so-called classical bacterium with rigid cell walls has occurred in the laboratory, presumably because ingredients in standard media suppress the L form (21, 22).

This paper is concerned with the requirements and activities of spontaneous L forms appearing in culture. It was found that the variant could adapt to marked nutritional changes, developing simultaneously with cocci on enriched media, or independently in media nutritionally deficient for cocci.

The early concept that an L form produces only the same enzymes as the bacillary type and in lesser amounts has been contradicted. The liquefaction of agar substrate can be a unique metabolic activity of the L variant, as shown here for the staphylococcus, and by others for *Clostridium welchii* (13).

The original concept that L variants are nonpathogenic has been modified by finding many examples of invasive ability or toxin production in *Clostridium tetani* (28), *Streptococcus pyogenes* (17), *Streptococcus viridans* (11), *Vibrio comma* (23, 32), proteus (23), and *Salmonella typhimurium* (15). Pathogenic L strains may be distinguished from nonvirulent strains by cytopathogenicity in tissue cultures employed for their growth (15).

Methods

Cultures

The 20 strains of *Staphylococcus aureus* were cultured from infections in

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children. Fourteen were freshly isolated in 1959. Six were Oeding strains isolated in 1953 (27).

Each strain was grown separately in tryptose phosphate broth at 37° C. After 18 hours, each culture was harvested and washed three times in 0.85% saline. Serial dilutions were prepared and standard plate counts were made to estimate the bacterial population.

Media

The *basal medium*, a modification of one used by Medill and O'Kane (22), had the following composition: glucose, 0.1%; sodium lactate, 2.0%; K_2HPO_4, 0.9%; salts, 0.2%; casamino acids, vitamin-free, 0.5%; sucrose, 10.0%; agar (Noble's), 0.8%. The triturated salt mixture was composed of $MgSO_4.7H_2O$, 40 g; NaCl, 2 g; $FeSO_4.7H_2O$, 2 g; $MnSO_4.4H_2O$, 8 g.

The agar was prepared separately in double strength and sterilized at 121° C for 15 minutes. The remaining components of the medium were prepared in double-strength solution and filter-sterilized. The two sterile portions were then aseptically combined.

Sucrose was added to produce hypertonicity, facilitating survival of protoplasts, wall-deficient forms (14, 16, 36). The *complete medium* consisted of the basal medium plus the following nutrilites: nicotinamide, thiamine, pyridoxine, biotin, and uracil, each in a final concentration of 1 μg/ml.

Duplicate studies in which the nutrilites were added in final concentrations of 10 μg/ml, or 100 μg/ml, gave findings similar to those with 1-μg concentrations, and are not presented in the results.

The nutrilites were chosen in accordance with information concerning requirements for coagulase-positive and coagulase-negative staphylococci (2, 10).

When quantitation of growth was desired, both the *basal* and *complete* media were employed as liquids, omitting the agar.

Incubation was at 37° C, anaerobic by the Fortner method (8). Incubation under 10% CO_2 tension gave similar results.

Cultural and Optical

To determine the rates and types of growth, cultures were inoculated into liquid media, incubated, and standard plate counts made in yeast dextrose agar. Additionally, morphological changes were observed by phase microscopy and with Gram-stained smears of centrifuged aliquots.

Tests for Coagulase Production by the L Form

Preliminary trials indicated that 200 units/ml of penicillin G potassium was the concentration at which maximum L-form growth was obtained without concurrent growth of staphylococci. The penicillin was added to a medium consisting of: nutrient broth (Difco), 0.8%; Noble's agar, 0.1%; sucrose, 2.0%; horse serum (Difco), 1.0%; supplement C (Difco), 1.0%. Incubation was in flasks shaken at room temperature for 18 hours. To test for coagulase, 0.5 ml of an 18-hour culture was mixed with an equal volume of fresh human plasma which had been pretested for coagulability with a known strain of *S. aureus*. Coagulase activity was estimated by arbitrarily judging the extent of the clot.

Results

Complete medium supported the growth of both the classical and L forms of staphylococci. As growth factors were sequentially removed, L forms became progressively more predominant, until, in the basal medium, only L forms were present.

Nutrition Versus Growth Morphology

When a large inoculum was introduced into the *complete medium* both the classical and L forms of staphylococci developed (see Table I). The L-form growth was typical of the progressing L cycle, i.e. filamentous and branching. In contrast, as the table indicates, when the basal medium was *not* reinforced with nutrients, colonies of the filamentous L growth occurred without accompanying staphylococci. Without added growth factors no cocci could develop even with an inoculum of 30,000 cocci per ml of medium. Thus L growth requirements were less than for the coccal stage.

TABLE I

The development of the L cycle in media deficient for the coccal stage

Inoculum of staphylococci	No growth factors added	Nicotinamide	Nicotinamide, biotin, pyridoxine, thiamine	Nicotinamide, biotin, pyridoxine, thiamine, uracil
3×10^{3}	L colonies	L colonies	L colonies	L colonies
3×10^{1}	L colonies	L colonies Staphylococci	L colonies Staphylococci	L colonies Staphylococci

When relatively few staphylococci were used as inoculum, the L form always developed exclusively. With such small inocula L variation progressed only to the spheroplast stage, the initial change of a bacterium to a swollen wall-deficient globule. Furthermore, the full complex of nutrilites did not succeed in boosting the growth beyond the spheroplast stage; increased nutrition resulted only in more-extensive multiplication of spheroplasts. In order to count the organisms the cultures were made in liquid media. Progressive changes in the organisms were followed at 2-hour intervals by examining and culturing aliquot samples. The data in Fig. 1 indicate that 250 staphylococci yielded 58,000 spheroplasts in the basal medium, or nearly 500,000 spheroplasts if the medium was reinforced with nicotinamide, biotin, pyridoxine, thiamine, and uracil. Similar results were obtained with even smaller inocula (1–5 cocci/ml). It is interesting that the complete medium, containing all the known growth factors for staphylococci, produced only spheroplasts if the inoculum was modest.

Continued Growth of L Forms in Subculture

In the basal medium the spheroplasts were only about three times as large as classical staphylococci. When plated in yeast dextrose agar an unexpected expansion occurred. The resultant enormous spheres, with detailed structure, are shown in Fig. 2. These bodies reached their maximum size within 24 hours

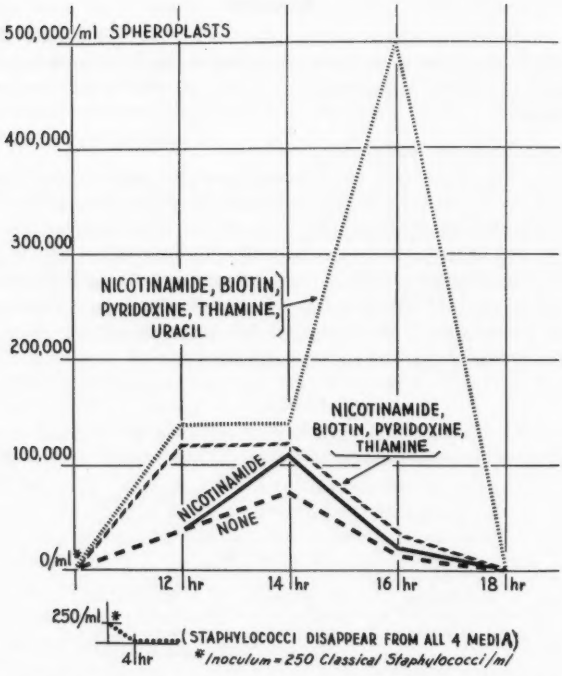


FIG. 1. Stimulation of spheroplast growth by vitamins and nucleotides.

and did not undergo further development. Subcultures made from the original spheroplast cultures after 18 hours resulted in no growth, correlating with the microscopic examination of the fluid which revealed that lysis of the bodies had occurred. The stimulus for the giantism is yet to be defined, but it may be produced by the products of carbohydrate fermentation, as is true for the "swollen L bodies" in P.P.L.O. (9).

For subculturing both the spheroplast and filamentous L forms, yeast dextrose agar was most suitable. Typical staphylococcal L colonies are shown in Fig. 3 *a* and *b*.

The growth of L forms, or development of the large spheroplasts, was not observed when tryptone glucose agar was used as a subculturing medium. Preliminary studies suggested that the inhibitor in this medium may be tryptone.

For continuous subculture of L forms, sucrose was not needed. Even initially sucrose was not mandatory as a few L forms consistently appeared when sucrose was omitted from the basal medium. The finding that the L form can be initiated without hypertonicity and without antagonists lends support to the idea that staphylococcal L forms can occur spontaneously *in vivo*.

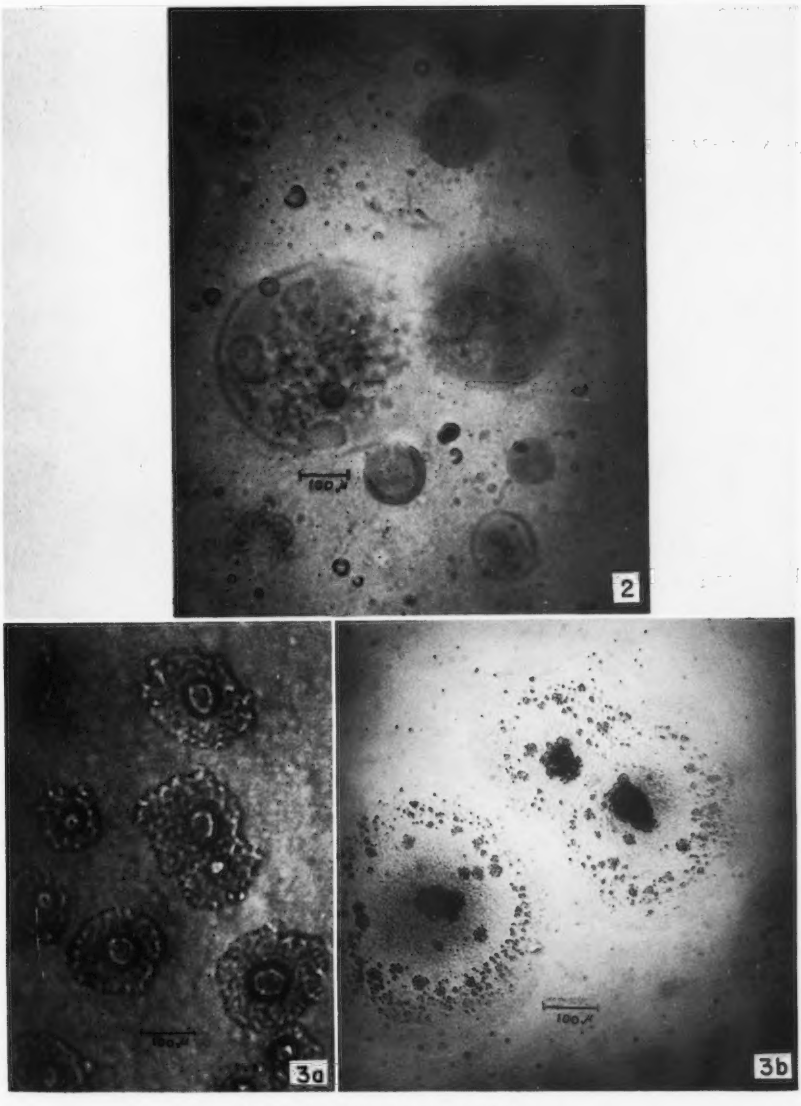
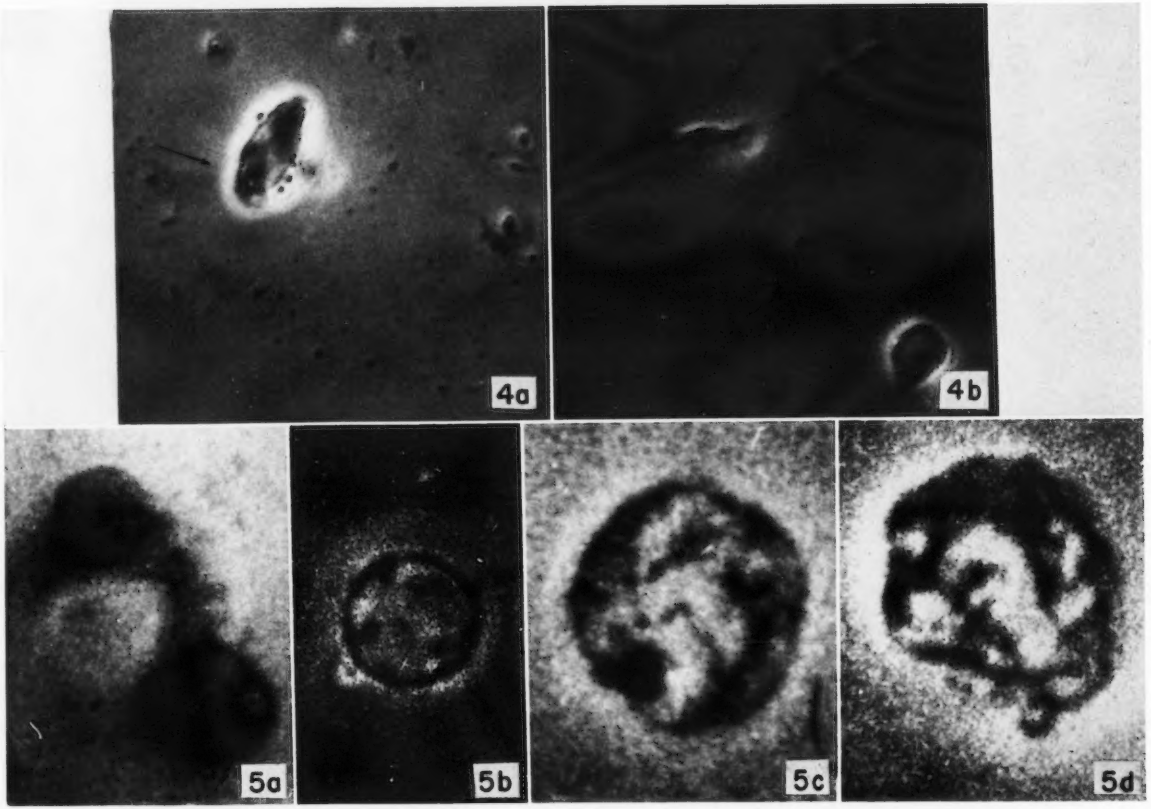


FIG. 2. Giant spheroplasts of staphylococci.
FIG. 3. L colonies of staphylococci.



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Reversion of L Forms to Staphylococci

Reversion to staphylococci occurred only in cultures containing blood. Reversion occurred easily with forms from the solid experimental media, usually being complete in 24 hours. From liquid media approximately 5% reversion took place in 24 hours, and continued incubation for 5 days made little change. A recent publication suggests that more reversion might have occurred if the L-form cultures had been subjected to prolonged incubation before being subcultured (31).

Coagulase Formation

In the tests for coagulase production by the L forms, penicillin was employed to assure lack of multiplication of the classical stage. Twelve strains of *S. aureus* in both the classical and L form of growth were examined. Coagulase production was demonstrated by each. The ratio of enzyme from classical and L form was estimated and varied with the strain, as detailed in Table II.

TABLE II

Coagulase activity from classical and L forms of *S. aureus*

Strain	Classical form	L form
0	4+	3+
1	4+	1+
1A	4+	2+
2	4+	1+
3	4+	1+
4	4+	2+
153	4+	3+
171	3+	3+
311	4+	2+
411	2+	2+
412	4+	1+
413	4+	2+

The classical form apparently produced more coagulase than the L form. Whether this difference is more apparent than real awaits further studies. Quantitative measurements were not performed in this study because of the inherent differences in the morphology of the classical and L forms of staphylococci. For the present, coagulase activity by the L stage has been demonstrated but no accurate quantitative comparison has been made.

L Forms Associated with Erythrocytes

An affinity of the L form for red cells was evident when cultures in medium containing blood were examined by phase microscopy. To ascertain whether the apparent intracellular position of the L forms was an optical illusion, portions of the cultures were embedded in paraffin, sectioned, and stained with Giemsa's stain. Erythrocytes caught in cross section showed L forms at varying levels within the red cell (see Fig. 4 *a* and *b*). Small spheroplasts, expanding L forms, and filaments, all showed this tropism. Approximately

FIG. 4. L forms within and on the surface of erythrocytes, shown in cross section.
FIG. 5. Erythrocytes with intracellular staphylococcal L bodies and branching filaments. Varying magnifications.

2% of the L forms were intracellular; however, extensive hemolysis in the media rendered the primary relationship unknown.

The surface appearance of erythrocytes with L forms is shown in Fig. 5.

Liquefaction of Agar

Lysis of agar media was often marked, proving to be a function of growth of the L stages, and never exhibited by cocci alone. Agar concentrations from 1.0% to 1.4% were liquefied, at times along the line of growth, in some cultures involving the entire plate. Some strains were judged to be more active in this respect than others. Lysis of agar by L variants of *Clostridium welchii* has been reported (13).

Discussion

Previous studies of L forms of staphylococci have emphasized the utilization of penicillin to induce this type of growth (6). L forms develop because the antibiotic prevents cell-wall synthesis from the precursor uridine diphosphoacetylmuramic acid peptide (30). The modified cell walls which result are depicted in the electronographs of Murray, Francombe, and Mayall (26).

It appears, however, that artificial interruption of cell-wall formation is not necessary to cause growth of the variant. The evidence presents two concurrent pathways maintaining a balance between L and typical growth, which is disturbed when either cycle is suppressed. Many components of standard media block development of the L stage. Thus, in the past, spontaneous L growth has been seen in a few species only, most notably *Streptobacillus moniliformis*. Medill and O'Kane (22) were the first to notice how markedly the L stage was inhibited by factors added to enrich media, e.g. peptone, yeast extract, and riboflavin. This laboratory has extended the ideas they initiated, finding that agar is toxic unless purified (21) and that serum must be limited in concentration (18, 21). When care is taken to avoid such inhibitors, L growth appears almost routinely from the genera investigated: staphylococci, streptococci (33), salmonella (20), and mycobacteria (21). No antimicrobial agent is necessary. Sucrose aids the survival and development of the spheroplast, but some L growth is established without hypertonicity. Thus on medium which permits rapid growth of the bacterial form, L growth can be concomitant in varying proportions, depending on the medium and the genus. By removing special factors required by the bacterial stage, pure culture of the L variant is assured. These principles are illustrated by the present work with staphylococci and have been published in studies with five species of mycobacteria (19, 21).

Certain questions arise which cannot as yet be answered. For example, why does the L form grow in a medium which will not support the growth of the classical form? And conversely, why is the L form not found on many common media used for staphylococcal growth? With identical inocula the presence of vitamins determined whether the L form alone developed or whether the classical coccal stage also appeared. Thus it seems that in the presence of vitamins a more-dependent type of metabolism is possible which results in cell-wall synthesis. In the absence of these special nutrilites the morphology resulting is entirely of the L form. In other words, the meta-

bolic pathways of the L forms may be capable of bypassing vitamins. It has been shown that essential amino acids are treated very differently by the P.P.L.O. than by bacteria (29). This fact, of course, is pertinent only if one believes in the essential identity of the P.P.L.O. and L variants.

Why does the classical form of staphylococcus thrive in certain media which suppress L growth? A possible explanation is that the media possess toxic molecules which are prevented from entering the microoganism only if it has the complete wall. It has been shown that molecules with a molecular weight of 10,000 or more cannot penetrate the staphylococcal wall (24). If the impurities in commercial agar which inhibit the L form are large molecules and fat-soluble they would be excluded by the wall but could penetrate the the cell membrane because of its lipid composition (24). The identities of the toxic components in peptone, some preparations of yeast extract, and concentrated serum likewise remain to be defined.

It might have been expected that the presence of a normal cell wall would facilitate growth in low-vitamin medium, since bacterial cell walls are known to concentrate lysine and promote its transportation to an intracellular position (4). The present data show, however, that the L form is selectively favored by minimal nutrition.

This paper emphasizes a point given inadequate attention in physiologic studies of the staphylococcus, namely, that the nutrilites carried endogenously by the inoculum may be more important than the composition of the culture medium. Thus, the complete medium used in this study should consistently support the growth of the classical form of the staphylococcus since it contained all the known necessary nutrilites. The fact that it did not suggests that the medium is adequate only when sufficient inoculum is employed to supply an endogenous depot of trace additives.

Intracellular growth of staphylococcal L forms in erythrocytes is analogous to a characteristic of streptococci. *In vivo* attachment of streptococcal L forms to erythrocytes has been reported by Vigouroux and Hannoun (35), who concluded their streptococcal L bodies were similar to Mulé's (25) intraerythrocytic particles found in blood of scarlet fever and rheumatic fever patients.

The current studies of sectioned erythrocytes, demonstrating that the staphylococcal L form grows intracellularly, adds another protozoanlike characteristic to this type of variant which has one mode of reproduction similar to merozoite formation in Plasmodia.

Intraerythrocytic growth of L organisms sharply distinguishes them from the bacterial stage, as the only bacterium known to infect the erythrocytes of man is *Bartonella bacilliformis* of Peruvian fever. The possible relationship of the intraerythrocytic stage to infection latency comprises an interesting subject for future investigation.

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NEW COMPLEMENT FIXING ANTIGEN FOR SERODIAGNOSIS OF GONORRHOEA[1]

N. A. Labzoffsky and A. E. Kelen

Abstract

A new method of preparing gonococcal antigen for use in the complement fixation test is presented. Briefly, the procedure consists of extracting *Neisseria gonorrhoeae* cells with pyridine and then exposing the washed sediment to ultrasonic treatment.

The new method is superior to that of Price by yielding highly potent, genus-specific and stable antigens, which are free of anticomplementary properties and possess a broader antigenic valence and a longer range of working antigenic power. It is simpler and more practical than Torrey's modification of the Price method since the lengthy and laborious procedure of strain selection is eliminated.

In routine use for the past several years, the new antigen has proved to be specific and reliable in the complement fixation test. The test itself provides a useful supplementary aid to the laboratory diagnosis of gonorrhoea.

Introduction

The laboratory diagnosis of gonorrhoea is based mainly on bacteriological methods by demonstrating the presence of the causative agent, *Neisseria gonorrhoeae*, in specimens from patients suspected of suffering from gonorrhoea. Quite often, however, especially in subacute, chronic, or complicated cases, microscopic examination of smears or cultivation attempts may not be successful and the demonstration of specific antibodies in the patient's serum by complement fixation may be required to establish the diagnosis (5).

The reliability of the complement fixation test (CFT) in the serodiagnosis of gonorrhoea depends mostly on the properties of the complement fixing antigen used. During the past fifty years, many different methods (2, 3, 6, 7, 8, 9, 10, 12) have been proposed for the preparation of the antigen, but none of them proved to be satisfactory in all respects. Among the various procedures recommended, the method of Price (9, 10) or its modification by Torrey (12) is usually considered the best and has been generally accepted for routine use during the past two decades.

The Price antigen is a fine colloidal suspension obtained by treating the gonococcus culture with $N/1$ NaOH, precipitating the proteins with $N/1$ HCl, and resuspending the precipitate with $N/10$ NaOH solution. Unfortunately, however, the majority of gonococcus strains treated this way yield antigens which are too highly anticomplementary for use and may give false positive reactions. On the other hand, individual strains selected for their relatively low anticomplementary activity may lack in breadth of antigenic valence and give rise to false negative results.

Torrey (12) attempted to overcome these difficulties by a lengthy and

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laborious procedure of selecting combinations of strains which would have wide antigenic valence and, at the same time, be low in anticomplementary properties. It was with the object of finding a more simple, but equally efficient method for the production of satisfactory gonococcal antigens that this study was undertaken.

Materials and Methods

Gonococcus Strains

Strains of *Neisseria gonorrhoeae* used in this study were freshly isolated cultures from specimens submitted to our laboratory for bacteriological examination. The isolates were checked for purity as well as for the possession of typical morphological, cultural, and biochemical characteristics by repeated serial passages from single colonies, by microscopic examination of individual colonies and stained smears prepared from the latter, and by the use of the oxidase reaction (5). Stock strains were kept alive by passaging twice weekly on solid culture media.

Cultivation of Gonococci

As culture medium, Mueller–Hinton blood agar (1) or Peizer agar plates (13) were used. A heavy suspension of a gonococcus strain was spread over the surface of 25–30 plates and incubated at 37° C for 2–3 days in an atmosphere containing 10% CO_2. Before harvesting the growth, each plate was examined under the microscope for cultural characteristics and for purity. Only plates showing typical growth and no contamination were scraped. The harvested growth of a strain from a number of plates was pooled and suspended in physiological saline.

Preparation of the Antigen

The crude saline suspensions of individual strains were adjusted to approximately the same density and each batch divided into two equal parts. One half of each batch was treated according to the Price method as modified by Torrey (12) and henceforth referred to as the Price–Torrey method. The other half was processed by the new method.

The new method of preparing gonococcal complement fixing antigens is schematically presented in Fig. 1. The crude saline suspension of a gonococcus strain was centrifuged at 3000 r.p.m. for 20 minutes, the sediment was washed twice in physiological saline, then resuspended in pyridine[2] to the original volume and kept at room temperature for 2 hours with occasional shaking. The pyridine extract was then removed by centrifugation at 3000 r.p.m. for 20 minutes and the sediment washed four times with physiological saline. Next, the sediment was resuspended in physiological saline to the original volume and then exposed to ultrasonic vibration[3] for five 2-minute periods. To avoid overheating, the material was thoroughly chilled between the periods of vibration. The disintegrated "whole" antigen so prepared was found to be stable for at least 1½ years at 4° C.

[2] C_5H_5N—79.10, British Drug Houses Ltd.

[3] The ultrasonic generator employed has a frequency of 500 kc and delivers to the crystal 500 acoustic watts.

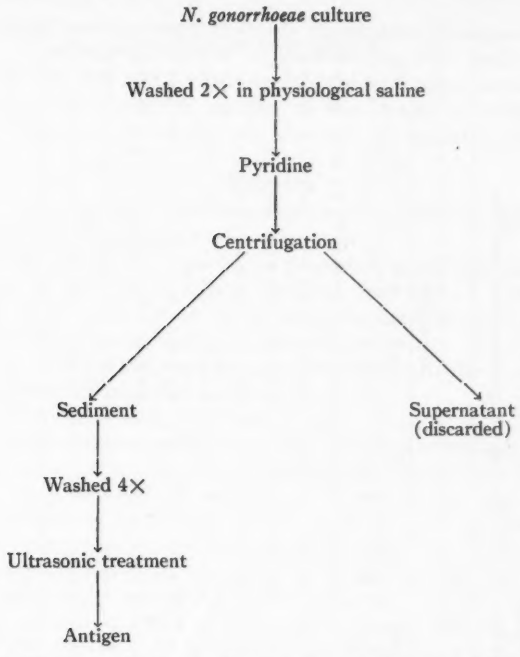

FIG. 1. Diagram of basic procedures in preparing gonococcal C.F. antigen.

Sera

The sera employed were hyperimmune rabbit sera and human patients' sera submitted to our laboratory for serological diagnosis. Rabbit immune sera were produced by intravenous injection of live gonococcus organisms given once a week for 5 weeks. The concentration of the organisms in the inoculum corresponded in density to that of No. 3 McFarland nephelometer tube and the doses were 0.25 ml, 0.5 ml, 1 ml, 2 ml, and 2.5 ml respectively. The animals were bled 2 weeks after the last injection. As controls, presumably normal human and rabbit sera, syphilitic human patients' sera, and commercial preparations of antimeningococcal hyperimmune rabbit sera were used. All sera were kept frozen at −20° C and inactivated at 56° C for 30 minutes prior to use.

Complement Fixation Test

The technique was similar to that described in an earlier publication (4). The units of antigen and antiserum respectively were defined as the highest dilution giving, in the presence of the other reagent, complete fixation of two units of complement. Two units of antigen were used for titration of antiserum and four units of antiserum for titration of antigen, two units of complement being used in each case. Each reagent was contained in 0.2 ml. Incubation was for 75 minutes in a water bath at 37° C.

TABLE I

Titration of gonococcal complement fixing antigens for potency and anticomplementary qualities

Antigen from strain No.	Titers of:												
	Price antigens						New antigens						
	1:8	1:16	1:32	1:64	1:128	1:256	1:8	1:16	1:32	1:64	1:128	1:256	
1	++++	++++	++++	++++	+++	−	++++	++++	++++	++++	++++	+++	T
	++++	+++	+	−	−	−	−	−	−	−	−	−	C
2	++++	++++	++++	++++	++	−	++++	++++	++++	++++	++++	++++	T
	+	−	−	−	−	−	−	−	−	−	−	−	C
3	++++	++++	++++	+	−	−	++++	++++	++++	++++	++++	++++	T
	−	−	−	−	−	−	−	−	−	−	−	−	C
4	++++	++++	++++	++++	++++	−	++++	++++	++++	++++	++++	++	T
	++++	++++	++++	++++	−	−	−	−	−	−	−	−	C
5	++++	++++	++++	++++	+++	−	++++	++++	++++	++++	+++	−	T
	++++	+++	−	−	−	−	−	−	−	−	−	−	C
6	++++	++++	++++	++++	++++	−	++++	++++	++++	++++	++++	+++	T
	+++	−	−	−	−	−	−	−	−	−	−	−	C
7	++++	++++	++++	++++	++++	−	++++	++++	++++	++++	++++	+++	T
	++++	++++	++++	++++	−	−	−	−	−	−	−	−	C
8	++++	++++	++++	++++	+	−	++++	++++	++++	++++	++++	++++	T
	++++	−	−	−	−	−	−	−	−	−	−	−	C
9	++++	++++	++++	+	−	−	++++	++++	++++	++++	++++	+++	T
	++++	++++	++++	−	−	−	−	−	−	−	−	−	C
10	−	−	−	−	−	−	++++	++++	++++	++++	+++	−	T
	−	−	−	−	−	−	−	−	−	−	−	−	C

NOTE: T = test; C = antigen control.
Hyperimmune rabbit serum No. 102 used in a dilution of 1:20.

The haemolytic system was 0.2 ml of 2% sheep cell suspension sensitized by 2½ units of amboceptor in an equal volume. The usual controls were always included in each test and the tests were read after serum and antigen controls were completely clear, usually 20 minutes after addition of haemolytic system. For the sake of brevity, controls are omitted from the tables.

Results

Comparison of Potency and Anticomplementary Properties of Antigens Prepared by the Two Methods

To compare the antigens prepared by the new method described above with those obtained by the Price–Torrey method, two sets of experiments were performed. In each case, the antigens were prepared by both methods from each of 10 different gonococcus strains selected at random. The results obtained in both sets of experiments were practically identical. The results of one set of the experiments are recorded in Table I.

From this table, it is seen that five antigens (Nos. 1, 4, 5, 7, and 9) prepared by the Price–Torrey method are too anticomplementary: their working antigenic power, i.e., the margin between the titer of the antigens and their anticomplementary property, is too narrow to permit their use in CFT where two units of antigen are used. The antigen prepared from strain No. 10 failed to exhibit any antigenicity even in the starting dilution of 1:8. On the other hand, antigens prepared from the same 10 strains by the new method were found to be completely free of anticomplementary activity and gave higher titers ranging between 1:128 and 1:256 as compared with the Price–Torrey antigens with titers from 1:32 to 1:128. Even the antigen from strain No. 10 revealed a relatively high titer of 1:128 when prepared by the new method. Similar results were obtained in the second set of experiments not recorded here for the sake of brevity.

On the whole, of a total of 20 strains tested, all gave antigens free of anticomplementary activity and of high potency, when the new method was used, whereas 55% of the same strains yielded antigens unsuitable for use when the Price–Torrey method was employed. This percentage is in a very close agreement with that found by Torrey (12), who observed that 13 out of 23 (56.6%) of the gonococcus strains tested gave antigens too highly anticomplementary for use in CFT, when prepared by their method.

Breadth of Antigenic Valence

The breadth of valence of the antigens prepared by the new method was tested by the use of immune rabbit sera and sera of human patients with a clinical diagnosis of suspected gonorrhoea. The results of testing three different immune rabbit sera (Table II) with homotypic and heterotypic gonococcal antigens showed that homotypic titers were somewhat higher, but not significantly so, the difference being only one dilution, if the last four-plus reaction is taken as the end point of titration. These results indicate the preponderance of common group-specific antigenic factors in all four strains involved.

Human patients' sera from different cities were tested with one monovalent antigen prepared from a single strain and two polyvalent antigens. One of the latter was made up of 5 strains (including the former single strain) and the

other was composed of the same 5 strains and an additional 5, a total of 10 strains. The results of this test (Table III) showed that 8 of the 16 sera tested failed to react with the monovalent antigen. Five of these gave positive reactions in titers of 1:8 to 1:16, when tested with the polyvalent antigen containing 5 strains, and all of them reacted with the other polyvalent antigen containing pool of 10 strains. In most cases, the titers with the polyvalent antigens were somewhat higher.

Polyvalent antigens prepared by the Price–Torrey method from the same strains were too anticomplementary for use and consequently comparative results could not be obtained.

Comparison of Specificity of Antigens Prepared by the Two Methods

Monovalent and polyvalent (5 strains) antigens were prepared by the

TABLE II

Results of complement fixation reactions with homotypic and heterotypic antigens

Antigens from strain No.	Immune rabbit sera produced by strain No.	With serum dilutions of:					
		1:16	1:32	1:64	1:128	1:256	1:512
174	449	++++	++++	++++	++++	++++	++
449	449	++++	++++	++++	++++	++++	++++
541	449	++++	++++	++++	++++	++++	+++
174	541	++++	++++	++++	++++	++++	++
449	541	++++	++++	++++	++++	++++	+++
541	541	++++	++++	++++	++++	++++	++++
174	1210	++++	++++	++++	++++	++++	+++
449	1210	++++	++++	++++	++++	++++	+++
541	1210	++++	++++	++++	++++	++++	++

TABLE III

Results of testing human patients' sera with monovalent and polyvalent antigens

No.	Origin of sera	Bacteriological findings	Titer of sera with antigen from:		
			One strain	Five strains	Ten strains
1	City A	0	–	8	8
2	City B	0	8	8	16
3	City B	0	32	64	64
4	City B	0	128	256	256
5	City B	0	128	128	128
6	City B	0	128	128	128
7	City B	0	8	16	16
8	City C	0	–	8	16
9	City D	0	–	8	16
10	City E	0	–	8	16
11	City F	+	32	32	32
12	City F	+	–	–	8
13	City F	+	–	–	8
14	City F	+	8	16	32
15	City F	+	–	16	16
16	City F	+	–	–	8

NOTE: 0 = no data available; + = *N. gonorrhoeae* isolated; – = negative in the starting dilution 1:8. Titers are expressed as reciprocals of serum dilutions.

Price–Torrey (12) and by the new methods from strains selected for their lack of anticomplementary property by the Price–Torrey method. These were tested against antimeningococcal immune rabbit sera, syphilitic human patients' sera, presumably normal human and rabbit sera, in addition to antigonococcal immune rabbit and human patients' sera. In Table IV results obtained with representative sera only are recorded. All four antigens were found to react with antigonococcal rabbit serum, gonococcal human serum, and antimeningococcal rabbit serum, but not with normal or syphilitic human serum nor with normal rabbit serum.

TABLE IV

Specificity of the antigens

Sera	Titers of sera tested with antigens			
	Strain No. 12		Pool of 5 strains	
	Price	New	Price	New
Antigonococcal immune rabbit serum No. 102	512	256	256	512
Gonorrhoea-positive human serum No. 1985	64	128	64	256
Antimeningococcal immune rabbit serum	64	8	128	64
Syphilis-positive human serum No. 2305	–	–	–	–
Normal rabbit serum	–	–	–	–
Presumably normal human serum	–	–	–	–

NOTE: Titers are expressed as reciprocals of serum dilutions.
– = negative in the starting dilution of 1:8.

Routine Use of the New Antigen for Serodiagnosis of Gonorrhoea

The new antigen was introduced in our laboratory for routine serodiagnosis of gonorrhoea in 1956. During the past five years, more than 500 serum specimens submitted to our laboratory for gonococcal CFT were tested routinely with this antigen (Table V). Ninety-six of them, approximately 18%, proved to be positive with titers ranging from 1:8 to 1:256. Most of the specimens tested were from patients clinically diagnosed as suspected to have gonorrhoea, but without bacteriological verification.

TABLE V

Results of complement fixation test in serodiagnosis of gonorrhoea during 1956–1960

Total no. of sera tested	Number of positive sera with a titer of 1 in:						Total no. of positive sera	% of positive sera
	8	16	32	64	128	256		
528	23	24	21	17	10	1	96	18.2%

Comments

On the basis of the data presented, it can be stated that the method outlined above for the preparation of gonococcal complement fixing antigens has a number of advantages over the generally accepted method of Price as modified by Torrey (12).

One of the main advantages of the antigen is its lack of anticomplementary activity. Antigens prepared from nearly 50 different strains selected at random have showed no such activity even in the lowest dilution tested. On the other hand, nearly all batches of antigen prepared from the same strains by the Price–Torrey method have shown a greater or lesser degree of anticomplementary activity and, in about half, the anticomplementary activity was sufficient to preclude their use in CFT.

It is assumed that some substances responsible for the anticomplementary effect are extracted from the gonococcus cells by the pyridine treatment, without affecting deleteriously the antigenic material itself. The pyridine extracts obtained as by-products of antigen preparation were found to be anticomplementary to approximately the same degree as the corresponding antigens prepared by the Price–Torrey method from the same gonococcus strains. At the same time, the antigenic potency of the pyridine-treated antigens was equal to or usually greater than that of the Price–Torrey antigens prepared from the same strains. In addition the range of working antigenic power was significantly wider with the new antigen than with that of Price–Torrey.

The new method also proved to be superior to the Price–Torrey method in providing antigens of a broader antigenic valence. As a result of an extensive study on the antigenic structure of *Neisseria gonorrhoeae*, Wilson (14) found that gonococci contain a variable combination of group-specific and type-specific antigens. These are present either in overt or, very frequently, in masked form. It would seem that the pyridine treatment results in the unmasking of some of these latent antigenic factors and hence in enhancing the potency and breadth of valence of the antigen so treated.

The new method has the advantage over that of Price–Torrey in not requiring the complex procedure of selecting strains which are at once of wide valence and low anticomplementary activity.

Because of the variable antigenic structure of *Neisseria gonorrhoeae*, a complement fixing antigen prepared from a single strain may or may not detect the presence of antibodies induced in a patient by infection with another gonococcus strain. Therefore, however efficient a method might be in unmasking latent antigens, the use of a single strain (unless it contains all the known group-specific antigenic factors) cannot be recommended as a source of complement fixing antigen. Because of the broader reactivity of polyvalent antigen as shown in Table III, the use of pooled antigens prepared from several different strains is suggested for routine serodiagnostic purposes.

With respect to specificity little difference was found between the two antigens (Table IV). The genus-specificity of both of them was clearly established by negative results with the lowest dilutions of all *non-Neisserial* sera tested. In this respect, the lack of cross-reactions with syphilis-positive human sera seems to be especially important. The existence of an intrageneric antigenic relationship between *Neisseria gonorrhoeae* and certain other members of the *Neisseria* genus was demonstrated earlier by several authors (11). This intrageneric relationship should be borne in mind when positive results are obtained in testing human patients' sera for gonococcal infection

by CFT. In view of the clinical differences among infections with the various *Neisseriae*, diagnostic difficulties are not apt to arise.

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COLIFORM BACTERIA IN SEA WATER AND SHELLFISH

I. LACTOSE FERMENTATION AT 35.5° AND 44° C[1]

A. D. TENNANT AND J. E. REID

Abstract

Of 6452 coliform strains isolated from sea water and shellfish specimens by membrane filtration (MF) and Violet Red Bile (VRB) agar techniques, 4728 (73.3 per cent) fermented lactose with the production of acid and gas within 48 hours at 35.5° C; these cultures were incubated in Eijkman medium, in a water-bath at 44° C, for 48 hours. Gas-positive tests were recorded for 94.6 per cent of *Escherichia coli* type I strains and 37.7 per cent of *E. coli* type II strains; 6.7 per cent of the "non-faecal" strains (represented by 10 coliform biotypes) were also gas-positive at 44° C. The capacity of selected strains to ferment lactose at higher temperatures (44.5° to 46.0° C) was also studied.

A total of 1724 (26.7 per cent) of the cultures produced completely typical coliform colonies on MF membranes or VRB agar plates but were incapable of rapid lactose fermentation at 35.5° C on subsequent isolation. *Aerobacter* biotypes were predominant (77 per cent), and 47.4 per cent of the cultures liquefied gelatin. The probable effect of lactose-degraded coliform bacteria on coliform density estimates made by the MF technique is discussed.

Introduction

The ability of most coliform bacteria to ferment lactose at 35.5° C with the rapid production of acid and gas is a major differentiating character which forms the basis for many practical procedures in the field of water bacteriology. In North America the 35.5° C coliform index of pollution, as cited in American Public Health Association "Standard Methods" (14, 15), has long been the primary tool used in the bacteriological control of water and shellfish. Some attempts have recently been made to apply European techniques for the differentiation of "faecal" and "non-faecal" coliform bacteria through the use of elevated (43–46° C) incubation temperatures. On the other hand, the possible significance to public health of coliform bacteria which are incapable of rapid lactose fermentation at 35.5° C has virtually been ignored.

The value of 44° C lactose fermentation tests for determining the presence of *Escherichia coli* in water and shellfish has been cited extensively by British workers (4, 5, 9, 19). In assessing the fidelity of such tests for detecting *E. coli*, however, the frequency with which other 44° C gas positive coliform biotypes occur is of considerable importance. While some early reports claimed complete test specificity for *E. coli* with incubation temperatures of 44° to 46° C, it is now well established that other 44° C gas positive coliform biotypes do occur (7, 8, 17, 18, 19). *E. coli* type II (IMViC −+−−) and *Aerobacter aerogenes* type I are the two 44° C positive biotypes most often reported. Thomas *et al.* (19) summarized the data from 11 earlier investigations and concluded that neither of these biotypes occurs frequently in any habitat; 44° C positive *E. coli* type II ranged from none in human faeces to 3.7 per cent of the total coliform flora in soil, sewage, and water, and 44° C

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positive strains of *A. aerogenes* type I ranged from none in human faeces to 7.8 per cent in filtered and chlorinated water. In a study of 5794 coliform strains isolated from surface water supplies in the United States, Geldreich *et al.* (7) reported that 92.7 per cent of 1184 *E. coli* type I strains fermented E.C. medium at 45° C, while only 21.8 per cent of 174 *E. coli* type II cultures, and 7.8 per cent of 4436 cultures of 10 other coliform biotypes, were able to do so.

More-frequent isolations of 44° C gas positive coliform bacteria other than *E. coli* type I have been reported in other countries. In a study of water and human faeces in Norway, Henriksen (8) found that 97 per cent of 1714 cultures of typical *E. coli* type I, and 35 per cent of 1536 cultures of other coliform biotypes, were capable of gas production at 44° C in Eijkman medium. Authors working in tropical climates (1, 3, 13) have concluded that a 44° C test could be considered specific for distinguishing between faecal and non-faecal coliform biotypes only if it could be proved that 44° C positive *Aerobacter* biotypes were normal intestinal inhabitants.

Coliform bacteria which ferment lactose slowly or weakly at 35.5° C have been isolated in significant numbers from faeces (8, 11, 12), water (16, 18, 20), and shellfish (18). Stuart *et al.* (16) considered such strains to be attenuated, degraded, or devitalized with respect to lactose, and concluded that it may be dangerous to disregard slow or weak lactose fermenters when assessing water quality. Borman *et al.* (2) considered that the loss or retardation of the ability to ferment lactose marks one of the "way-stations" along an evolutionary path, a transition point between commensal and parasitic existence.

It is thus evident that coliform bacteria which do not conform to classical criteria in respect to lactose fermentation are widely distributed, and that the incidence of such biotypes in water varies considerably by area. The present study concerns the incidence of these "aberrant" coliform biotypes in Canadian shellfish-growing areas.

Methods

All of the cultures studied were isolated from sea water samples and oyster and clam specimens from various locations, with varying degrees of pollution, during studies of shellfish-growing areas conducted in Prince Edward Island and New Brunswick by the Laboratory of Hygiene. The Membrane Filter (MF) procedure (14) was applied to all sea water samples; all organisms that produced a dark colony with a metallic sheen after 20 ± 2 hours of incubation on Bacto–M–Endo MF broth at 35.5° C were considered as coliform bacteria. Shellfish specimens were collected and prepared for testing in accordance with the method outlined in the A.P.H.A. "Standard Methods for the Examination of Shellfish and Shellfish Waters" (15). Aliquots of sample were plated with Bacto – Violet Red Bile agar and incubated for 20 ± 2 hours at 35.5° C; purplish red subsurface colonies surrounded by a reddish zone of precipitated bile were considered as coliform bacteria.

Representative, typical discrete colonies were isolated from MF membranes and VRB plates, replated on Bacto–Tryptone Glucose Extract agar to ensure purity, and subjected to the following tests: lactose fermentation tests (Bacto–Lactose broth, 35.5° C, 21 days; Bacto–Eijkman medium, 44.0 ± 3° C, 48

hours); indol test (Kovacs; 35.5° C, 48 hours); methyl red test (Bacto–MR–VP medium, 30° C, 5 days); Voges–Proskauer test (Barritt's; Bacto–MR–VP medium, 30° C, 48 hours); citrate test (Bacto–Simmons Citrate agar, 35.5° C, 72 hours); and the gelatin liquefaction test (Bacto–Nutrient gelatin, 35.5° C, 7 days).

Results and Discussion

A total of 6452 cultures of coliform bacteria were studied (5080 from sea water, 1372 from shellfish). Of the 6452 cultures, 4728 (73.3 per cent) produced acid and gas from lactose broth at 35.5° C within 48 hours; the biochemical reactions given by these strains are recorded in Table I. Of the 2054 *E. coli* strains included, 87.8 per cent were 44° C gas positive. Of the

TABLE I

Biochemical reactions of 35.5° C gas positive coliform bacteria from sea water and shellfish

Coliform (IMViC) biotype	Number examined	44° C gas positive (Eijkman medium)		Gelatin liquefaction	
		No.	%	No.	%
++−− (*E. coli* I)	1810	1712	94.6	3	0.2
−+−− (*E. coli* II)	244	92	37.7	0	
Total "faecal"	2054	1804	87.8	3	0.1
++−+ (*C. freundii* II)	128	23	18.0	5	3.9
++++	50	9	18.0	0	
−−++ (*A. aerogenes* I)*	1213	114	9.4	154	12.7
++−−	11	1	9.1	0	
−++−	25	1	4.0	0	
+−++ (*A. aerogenes* II)	453	15	3.3	11	2.4
−+−+ (*C. freundii* I)	593	13	2.2	11	1.9
−+++	120	2	1.7	3	2.5
−−−+	6	1	—	1	—
−−+−	75	0		3	4.0
Total "non-faecal"	2674	179	6.7	188	7.0

*Includes strains of *Aerobacter cloacae*.

remaining 2674 cultures, representing 10 "non-faecal" coliform biotypes, 6.7 per cent were also 44° C gas positive. These data are in close agreement with those obtained by Geldreich *et al.* (7) in a study of coliform bacteria isolated from surface waters and subjected to the E.C. test at 45° C.

Since 94.6 per cent of *E. coli* type I cultures were 44° C gas positive, a test for lactose fermentation at this temperature is reasonably specific for this biotype. The test, however, is less specific for *E. coli* type II, the other coliform biotype generally regarded as "faecal". In some areas *Aerobacter* and *Citrobacter* strains capable of lactose fermentation at 44° C are a definite threat to the specificity of this test for *E. coli*; it has been our experience that the relative incidence of 44° C gas positive strains of these biotypes varies widely with area and season. Additional data on the incidence of 44° C gas positive "non-faecal" coliform biotypes in sewage and polluted water is definitely needed before the "faecality" of such strains can be determined.

Representative 44° C gas positive coliform cultures were retested in Bacto–E.C. medium at four temperatures in the 44–46° C range (Table II). An in-

TABLE II

Gas production in E.C. medium at 44–46° C by 44° C gas positive coliforms

Coliform (IMViC) biotype	Hours of incubation	Gas-positive at incubation temperature of:							
		44.0±0.2° C	44.5±0.2° C		45.0±0.2° C		46.0±0.2° C	
		(total)	No.	%	No.	%	No.	%
++−−	24	135	135	100	133	98.5	114	84.4
	48				134	99.3	128	94.8
−+−−	24	52	52	100	52	100	45	86.5
	48						48	92.3
+−++	24	69	63	91.3	61	88.4	50	72.5
	48		64	92.8	63	91.3	60	87.0
+++−	24	4	4	100	4	100	3	75.0
	48						3	75.0
++−+	24	10	9	90.0	9	90.0	7	70.0
	48		10	100	9	90.0	7	70.0
−−++	24	137	87	63.5	22	16.1	0	
	48		105	76.6	75	54.7	8	5.8
−+++	24	5	3	60.0	0		0	
	48		4	80.0	1	20.0	0	
−+−+	24	16	7	43.8	1	6.3	0	
	48		8	50.0	1	6.3	0	

crease in incubation temperature above 44° C tended to suppress some "non-faecal" coliform biotypes, particularly *A. aerogenes* type I and *C. freundii* type I. Of the 187 *E. coli* strains, only 5.9 per cent were incapable of gas production at 46° C. At the same time, a majority of the *A. aerogenes* type II (+−++), *C. freundii* type II (++−+), and +++− strains were 46° C gas positive. Thus, while some increase in incubation temperature, possibly to 45° C, may be desirable in the interests of increased test specificity, complete fidelity for *E. coli* cannot be achieved in this manner. It is also evident that water-bath incubation, with precise temperature control, is required if strictly comparable data are to be obtained.

Of the 6452 coliform cultures studied, 1724 (26.7 per cent) did not ferment lactose with the production of gas within 48 hours at 35.5° C; 998 of these strains were isolated from sea water (MF) while the remainder were from shellfish specimens (VRB agar). Many of the strains fermented lactose to some degree (75 with the production of gas after 3 days of incubation at 35.5° C; 214 with the production of gas after 4 to 7 days of incubation; 360 with gas production after 8 to 21 days of incubation; and 586 with the production of acid only at some time during the 3-week incubation period at 35.5° C). The biochemical reactions recorded for these strains are given in Table III.

TABLE III

Biochemical reactions of 35.5° C gas negative coliform bacteria from sea water and shellfish

Coliform (IMViC) biotype	Number of cultures examined	Incidence, %	Liquefaction of gelatin	
			No.	%
−−++	754	43.7	360	47.7
+−++	391	22.7	363	92.8
−−+−	147	8.5	106	72.1
−+−+	116	6.7	12	10.3
−+−−	92	5.3	37	40.2
++−−	78	4.5	32	41.0
++−+	64	3.7	53	82.8
−−−+	36	2.1	11	30.6
−++−	17	1.0	2	11.8
−+++	16	0.9	1	6.3
+++−	10	0.6	10	100
++++	3	0.2	2	66.7
Totals	1724		989	57.4

Lactose-degraded *A. aerogenes* biotypes (−−++, +−++, −−+−, −−−+) constituted a majority (77 per cent) of the cultures, while *E. coli* biotypes (++−−, −+−−) were definitely in the minority (9.9 per cent). Of particular interest was the very high incidence of gelatin-liquefying strains (57.4 per cent), as compared with the mean incidence of 4.0 per cent for the 35.5° C gas positive coliform strains reported in Table I.

The fermentation of lactose at 35.5–37° C is usually regarded as the essential attribute for the identification of coliform bacteria in the assessment of the sanitary quality of water. It is well known, however, that some strains ferment lactose slowly and others not at all, while in other respects they resemble

the more typical strains. Ewing and Edwards (6) conclude that the group to which a microorganism belongs must be determined by a combination of biochemical reactions, not by a single property. They state that each group of bacteria that ferments lactose promptly also includes counterparts that do not attack this substrate or do so only after prolonged incubation; failure to ferment lactose should therefore not exclude a culture from a group of which it is otherwise a typical member. The International Enterobacteriaceae Subcommittee does not recognize the genus *Paracolobactrum* nor the paracolobactrum group (10).

We regard the rarity with which lactose-degraded coliform bacteria have been reported in water supplies as a reflection of an evident lack of interest in these organisms, and of the almost universal use of standard density estimate tests based on rapid lactose fermentation. It has been our experience that lactose-degraded strains most commonly constitute circa 10 to 20 per cent of the total coliform flora of shellfish-growing water. Their incidence, however, can be much higher; in one study area, "aberrant" strains accounted for 63 and 78 per cent of the coliform bacteria isolated from sea water and oyster specimens respectively (18). The incidence of lactose-degraded coliform bacteria in shellfish-growing water increased with heavy rainfall and increased land-wash from the watershed, and appeared to increase with distance from the primary pollution source. This would tend to support the hypothesis of Stuart *et al.* (16) that coliform bacteria may become attenuated with respect to the fermentation of lactose after exposure to unfavorable environmental conditions.

The degree of sanitary importance which may be attached to lactose-degraded coliform bacteria is certainly open to question. It is evident, however, that such strains, when present in significant numbers in water, will have a marked influence on coliform density measurements made by membrane filtration and VRB agar plating techniques. It should be stressed that all of the aberrant coliform strains studied formed perfectly typical colonies on the Endo MF preparations and VRB agar plates. On this basis, it would be surprising if coliform density estimates obtained by the MF and standard tube-dilution (MPN) techniques were strictly comparable for all waters. It will continue to be difficult to relate the two indices precisely until much more is known about the sanitary significance of the various organisms of the coliform group and the aberrant strains which are included in density estimates made with the two techniques.

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COLIFORM BACTERIA IN SEA WATER AND SHELLFISH

II. THE E. C. CONFIRMATION TEST FOR ESCHERICHIA COLI[1]

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Abstract

Recently there has been considerable interest in Canada and the United States in the development of an effective, simple test for the estimation of *Escherichia coli* densities in shellfish and shellfish-growing waters as a better indication of "faecal" pollution than that provided by the coliform group. The fidelity of the E.C. confirmation test for the determination of *E. coli* densities was evaluated in 15 shellfish-growing areas which were classified as "polluted" or "unpolluted" according to generally accepted criteria. Coliform strains were isolated from 2765 E.C. gas positive confirmation test cultures and identified by IMViC tests. The mean fidelity of the test, as an indication of the presence of *E. coli*, was circa 90 per cent; contrary to expectations, however, the per cent recovery of *E. coli* was higher in unpolluted areas than in polluted areas, and varied considerably from region to region. *Aerobacter aerogenes* types I and II were the most common other E.C. gas positive coliform biotypes found. The usefulness of the confirmation test and the sanitary significance of these data are discussed.

Introduction

Bacteriological control of public health hazard in North American shellfish-growing areas has largely been based on the application of American Public Health Association "Standard Methods" (2, 3) tests for coliform bacteria capable of lactose fermentation at 35.5° C. Such control has been generally satisfactory; at times, however, it has been difficult in some growing areas to relate coliform densities to the presence or absence of positive pollution sources, particularly during periods of rainfall-induced run-off. The presence of *Escherichia coli* has generally been interpreted as positive proof of faecal contamination. The routine application of a test for this organism in shellfish and sea water has, however, awaited the development of an accurate, simple procedure which does not involve confirmation through pure culture isolation.

Selective examination for *E. coli* by means of elevated incubation temperatures (43° to 46° C) has received much study, and presumptive tests using an incubation temperature circa 44° C are widely used, particularly in Europe, for the determination of the bacteriological quality of water and shellfish. Recent studies, largely unpublished, indicate that a "confirmation" or "subculture" test for lactose fermentation at 44° or 44.5° C, in conjunction with the "Standard Methods" confirmed MPN test, would have considerable advantage over a direct presumptive test at 44–44.5° C. Tennant (4) reported very close agreement in estimates of *E. coli* (as a per cent of the total coliform flora of sea water and oysters) made by (*a*) a confirmation test using Bacto–Eijkman medium at 44° C, and (*b*) direct isolation and identification of representative coliform strains. Further study in the United States has culminated

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in the adoption of the E.C. (44.5° C) confirmation test as the sole coliform criterion of pollution and mishandling of shucked market oysters (1).

The present report concerns an evaluation of the fidelity of the E.C. confirmation test as a means of estimating *E. coli* densities, and of the degree of interference caused by "aberrant" *Aerobacter* and intermediate coliform strains.

Methods

A total of 15 shellfish-growing areas were studied (six on the north shore of the estuary of the St. Lawrence River in the Province of Quebec; nine in the Province of Prince Edward Island). The areas were classified as polluted or unpolluted through the application of all available bacteriological and public health engineering data. A total of 3530 sea water samples and 85 shellfish specimens were taken periodically from fixed sampling stations in the study areas. All specimens were tested within 6 hours of collection by the methods outlined in the recently revised A.P.H.A. "Standard Methods for the Bacteriological Examination of Shellfish and Shellfish Waters" (3); coliform confirmed most probable numbers (MPNs) per 100 ml or per 100 g of sample were determined (Bacto–Lactose broth, with five tubes in each of at least three dilutions, incubation at 35.5° C for 24 and 48 hours, and confirmation of gas-positive lactose broth cultures in Bacto–Brilliant Green Bile broth).

The presence of E.C. gas positive coliform strains was determined for all samples by the confirmation of all positive presumptive lactose broth cultures in Bacto–E.C. medium, with incubation for 24 hours in a serological water-bath equipped with a circulation pump and controlled at 44.5 ± 0.3° C. Results were expressed as most probable numbers of E.C. gas positive coliform bacteria per 100 ml or per 100 g of sample.

All E.C. gas positive cultures from representative samples from each study area were streaked on Bacto–E.M.B. agar, and the plates were incubated at 35.5° C for 24 hours. A discrete colony with metallic sheen was selected from each plate for transfer to Bacto–E.C. medium (incubation 44.5° C for 24 hours) and subsequent identification. If none of the colonies had metallic sheen, or if the first selected culture was E.C. gas negative, representative colonies were selected from the same plate for further study. All of the isolated cultures were subjected to the following biochemical tests: indol test (Kovacs; 35.5° C, 48 hours); methyl red test (Bacto–MR–VP medium, 30° C, 5 days); Voges–Proskauer test (Barritt's; Bacto–MR–VP medium, 30° C, 48 hours); citrate test (Bacto–Simmons Citrate agar, 35.5° C, 72 hours); and the E.C. test (Bacto–E.C. medium, 44.5° C, 48 hours).

Results and Discussion

A summary of the coliform, and E. C. gas positive coliform, MPNs obtained for sea water and shellfish specimens from the 15 study areas is given, at three percentile levels, in Table I.

The mean incidence of *E. coli* and other 44.5° C E.C. gas positive coliform strains in sea water, as estimated by the E.C. confirmation test and expressed as a percentage of the total coliform density, was generally lower in Quebec than in Prince Edward Island study areas (Table II). The mean coliform

TABLE I

Summary of percentile levels and coliform and E.C. gas positive most probable numbers in sea water and shellfish

Area	No. of samples	Most probable numbers per 100 ml					
		Coliform			E.C.		
		50%	90%	10%	50%	90%	10%
Q-1	174	290	1600	2	110	1600	1.8
Q-2	270	7.8	350	1.8	2	70	1.8
Q-3	246	79	1600	11	23	540	4
Q-4	282	11	220	1.8	2	22	1.8
Q-5	252	22	1600	2	7.8	350	1.8
Q-6	127	4.5	70	1.8	2	13	1.8
Clams	59	790	35000	68	330	8100	18
P-1	144	350	1600	49	130	1600	25
P-2	210	13	79	1.8	4.5	33	1.8
P-3	175	1.8	6.8	1.8	1.8	4.5	1.8
P-4	240	1.8	11	1.8	1.8	7.8	1.8
P-5	78	105	1600	6.8	29	920	2
P-6	480	2	17	1.8	1.8	11	1.8
P-7	96	70	920	13	33	240	4.5
P-8	396	4	23	1.8	2	17	1.8
P-9	360	4.5	23	1.8	2	13	1.8
Clams	12	1700	6000	45	790	4800	45
Oysters	14	78	390	20	20	220	18

TABLE II

Mean coliform: E.C. MPN ratios in sea water and shellfish

Area	Pollution status	Mean coliform:E.C. MPN ratios	
		All samples taken in area	Samples used in isolation study
Q-1	Gross	1:0.51	1:0.56
Q-2	Negligible–slight	1:0.42	1:0.70
Q-3	Slight–gross	1:0.35	1:0.37
Q-4	Slight–moderate	1:0.29	1:0.41
Q-5	Moderate–slight	1:0.37	1:0.47
Q-6	Negligible	1:0.45	1:0.65
Clams		1:0.45	1:0.49
P-1	Gross	1:0.44	1:0.43
P-2	Slight	1:0.49	1:0.66
P-3	Negligible	1:0.81	1:0.87
P-4	Negligible	1:0.60	1:0.77
P-5	Moderate–gross	1:0.51	1:0.51
P-6	Negligible	1:0.75	1:0.89
P-7	Moderate–gross	1:0.57	1:0.50
P-8	Negligible	1:0.77	1.0:83
P-9	Negligible–slight	1:0.66	1:0.83
Clams		1:0.73	1:0.75
Oysters		1:0.50	1:0.50

TABLE III

Incidence of 44.5° C E.C. gas positive coliform biotypes in E.C. gas positive confirmation test cultures for sea water

Coliform (IMViC) biotype	Area Q-1		Area Q-2		Area Q-3		Area Q-4		Area Q-5		Area Q-6		Area P-1		Area P-2	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
E. coli I (++−−)	117	90.7	113	91.1	160	82.9	180	83.7	169	80.5	90	91.8	99	84.6	203	90.6
E. coli II (−+−−)	2	1.6	3	2.4	6	3.1	3	1.4	5	2.4	0		2	1.7	5	2.2
No. cultures containing "faecal" biotypes	119	92.2	116	93.5	166	86.0	183	85.1	174	82.9	90	91.8	101	86.3	208	92.9
A. aerogenes I (−−++)	3	2.3	3	2.4	15	7.8	24	11.2	24	11.6	3	3.1	7	6.0	8	3.6
A. aerogenes II (+−++)	2	1.6	3	2.4	12	6.2	5	2.3	6	2.9	0		8	6.8	3	1.3
Intermed. I (−+−+)	1	0.8	0		1	0.5	2	0.9	2	1.0	0		1	0.9	2	0.9
Intermed. II (++−+)	2	1.6	1	0.8	2	1.0	0		3	1.4	0		2	1.7	1	0.4
Irregular V (−−+−)	1	0.8	0		0		0		0		0		0		0	
MR+VP+(−+++, +++−)	1	0.8	0		0		0		0		4	4.1	3	2.6	1	0.4
E.C. gas negative coliform biotypes	3	2.3	2	1.6	4	2.1	1	0.5	1	0.5	1	1.0	1	0.9	2	0.9
No. cultures which did not contain "faecal" coliform biotypes	10	7.8	8	6.5	27	14.0	32	14.9	36	17.1	8	8.2	16	13.7	16	7.1

Coliform (IMViC) biotype	Area P-3		Area P-4		Area P-5		Area P-6		Area P-7		Area P-8		Area P-9	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
E. coli I (++−−)	99	98.0	132	97.1	218	96.0	32	86.5	163	90.1	43	91.3	306	98.1
E. coli II (−+−−)	1	1.0	1	0.7	5	2.2	0		2	1.1	0		3	1.0
No. cultures containing "faecal" biotypes	100	99.0	133	97.8	223	98.2	32	86.5	165	91.2	43	91.3	309	99.0
A. aerogenes I (−−++)	2	2.0	1	0.7	2	0.9	1	2.7	7	3.9	2	4.3	6	0.6
A. aerogenes II (+−++)	0		3	2.2	2	0.9	0		2	1.1	2	4.3	1	0.3
Intermed. I (−+−+)	0		0		0		0		2	1.1	0		0	
MR+VP+(−+++)	0		0		0		0		1	0.6	0		0	
E.C. gas negative coliform biotypes	0		0		0		4	10.8	5	2.8	0		0	
No. cultures which did not contain "faecal" coliform biotypes	1	1.0	3	2.2	4	1.8	5	13.5	16	8.8	3	6.5	3	1.0

E.C. MPN ratios, as derived from the average of the coliform: E.C. MPN ratios for the individual samples, also appear to indicate that E.C. gas positive strains constitute a smaller percentage of the total coliform density in "polluted" than in "unpolluted" areas. These data are particularly striking in Prince Edward Island areas, where there was a greater degree of uniformity of pollution levels within each area.

The incidence of *E. coli* type I (IMViC ++−−), *E. coli* type II (IMViC −+−−), and other coliform biotypes isolated from E.C. gas positive confirmation test cultures is recorded in Tables III and IV. *E. coli* type I was isolated from 82.2 and 94.0 per cent of the E.C. gas positive cultures examined in Quebec and P.E.I. respectively. In Quebec the per cent recovery of *E. coli* type I strains was lower for shellfish specimens than for sea water samples; the reverse was true in P.E.I. *A. aerogenes* type I (IMViC −−++) and *A. aerogenes* type II (IMViC +−++) were the most common other E.C. gas positive coliform biotypes isolated.

Other E.C. gas positive coliform biotypes were found in association with *E. coli* type I in 28 E.C. gas positive cultures; similarly, E.C. gas negative coliform strains were isolated in association with E.C. gas positive strains from 61 E.C. cultures. The fact that mixed cultures do occur in E.C. gas positive tests would appear to preclude the direct use of other screening tests to further confirm the presence or absence of "faecal" coliform biotypes; if further confirmation is desired it will be necessary to plate the E.C. cultures and select colonies for further testing. It was also noted that the production of a characteristic metallic sheen on Bacto–E.M.B. agar is not a completely reliable criterion of the presence of "faecal" coliform biotypes; many "non-faecal" coliform strains produced typical sheen, while some *E. coli* strains failed to do so. Sheen was also shown to be a transient phenomenon with some *E. coli* type I strains.

It is difficult to explain our failure to isolate 44.5° C gas positive coliform strains from 37 (1.3 per cent) of the E.C. gas positive cultures. It is possible that minority strains responsible for gas production in a mixed flora may have been overlooked at the time of isolation from the E.M.B. agar plates. Most of the gas negative strains isolated from E.C. gas positive cultures had ++−− or −−++ IMViC reactions; these strains may have produced small amounts of gas in the E.C. confirmation test and failed to do so in pure culture. In any event, we contend that there can be no true "false positive" reactions under the terms of the E.C. confirmation tests.

The E.C. test had a lower fidelity when applied to presumptive lactose broth cultures which were not recorded as gas positive until after 48 hours' incubation. Such cultures, however, should be included in the test since *E. coli* was still the predominant coliform biotype in E.C. gas positive cultures derived in this manner.

It may be concluded that the E.C. confirmation test provides a reasonably efficient, simple method for the estimation of *E. coli* densities in sea water and shellfish, but that the precision of the method varies widely from area to area. Other E.C. gas positive coliform biotypes, particularly *A. aerogenes* types I and II, are a definite threat to the specificity of the test. These data have done little to define the faecal or non-faecal origin of these coliform biotypes,

except to demonstrate that they may be present in significant numbers in polluted sea water. It would appear that sewage entering the polluted study areas was characterized by a very wide distribution of coliform biotypes; this observation would tend to confirm the concept in which all 35.5° C gas positive

TABLE IV

Summary of incidence of *E. coli* and other 44.5° C E.C. gas positive coliform biotypes in E.C. gas positive confirmation test cultures

Incidence of coliform biotypes	All Prince Edward Island study areas				
	Polluted sea water	Unpolluted sea water	Clams	Oysters	Totals
Total no. E.C. gas positive cultures examined	200	1181	84	34	1499
Cultures containing E.C. + *E. coli* type I	174 (87.0%)	1121 (94.9%)	81 (96.4%)	33 (97.1%)	1409 (94.0%)
Cultures containing E.C. + *E. coli* type II	2 (1.0%)	17 (1.4%)	1 (1.2%)	0	20 (1.3%)
Cultures containing E.C. + *A. aerogenes* I	10 (5.0%)	22 (1.9%)	0	1 (2.9%)	33 (2.2%)
Cultures containing E.C. + *A. aerogenes* II	10 (5.0%)	11 (0.9%)	0	1 (2.9%)	22 (1.5%)
Cultures containing other E.C. + biotypes	6 (3.0%)	7 (0.6%)	0	0	13 (0.9%)
Cultures from which no E.C. + coliform biotype could be isolated	5 (2.5%)	7 (0.6%)	2 (2.4%)	0	14 (0.9%)

Incidence of coliform biotypes	All Quebec study areas				All study areas, totals
	Polluted sea water	Unpolluted sea water	Clams	Totals	
Total no. E.C. gas positive cultures examined	747	222	297	1266	2765
Cultures containing E.C. + *E. coli* type I	626 (83.8%)	203 (91.4%)	212 (71.4%)	1041 (82.2%)	2450 (88.6%)
Cultures containing E.C. + *E. coli* type II	16 (2.1%)	3 (1.4%)	3 (1.0%)	22 (1.7%)	42 (1.5%)
Cultures containing E.C. + *A. aerogenes* I	66 (8.8%)	6 (2.7%)	51 (17.2%)	123 (9.7%)	156 (5.6%)
Cultures containinge E.C. + *A. aerogenes* II	25 (3.3%)	3 (1.4%)	17 (5.7%)	45 (3.6%)	67 (2.4%)
Cultures containing other E.C. + biotypes	15 (2.0%)	5 (2.3%)	9 (3.0%)	29 (2.3%)	42 (1.5%)
Cultures from which no E.C. + coliform biotype could be isolated	11 (1.5%)	9 (4.1%)	3 (1.0%)	23 (1.8%)	37 (1.3%)

coliform biotypes are considered as presumptive evidence of faecal pollution. The demonstrated lower percentage incidence of *E. coli* in sea water known to contain sewage pollution, and the higher incidence of "faecal" coliform biotypes in relatively unpolluted areas, would tend to discredit the thesis upon which the use of the E.C. confirmation test as the sole test for bacterial pollution of Canadian shellfish-growing areas would be based.

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THE EFFECT OF CHLORIDES OF MONOVALENT CATIONS, UREA, DETERGENTS, AND HEAT ON MORPHOLOGY AND THE TURBIDITY OF SUSPENSIONS OF RED HALOPHILIC BACTERIA[1]

DINAH ABRAM[2] AND N. E. GIBBONS

Abstract

Suspensions of *Halobacterium cutirubrum*, grown and suspended in 4.0 *M* sodium chloride, showed on transfer to increasingly lower concentrations of sodium chloride an increase in turbidity followed by an abrupt decrease. When the suspensions were placed in potassium, rubidium, cesium, lithium, or ammonium chlorides, there was no increase in turbidity but usually a gradual decrease as the concentration of the salt is decreased. In potassium, rubidium, and cesium chlorides these changes were correlated with a change in morphology from rods, through transition forms, to spheres, similar to changes in NaCl, except that transition forms were found even at concentrations of 4.0 *M*. In lithium and ammonium chlorides there was an immediate change at 5.0 and 4.5 *M* respectively and two to five small spheres were formed from each rod. In mixtures of sodium and potassium chlorides the morphology of the cells depended on the ionic strength and concentration of sodium. Spheres were also produced by heating the cells to 60–70° C, the time required for the conversion increasing with increasing salt concentration. In urea solutions the cells lysed, although at certain concentrations of urea and NaCl a change to small spheres was observed. Ionic detergents caused the cells to disintegrate gradually. These observations are taken as further evidence that sodium is required to maintain the cell wall of the red halophilic rods.

Introduction

In a previous paper (1) the changes in the turbidity of suspensions of red halophilic rods and in their morphology with changes in the sodium chloride content of the suspending medium were attributed to changes in the structural material of the cells and it was concluded that the role of sodium chloride in the life of halophilic bacteria was not simply osmotic.

The work has now been extended to chlorides of other monovalent cations and mixtures of chlorides. A few substances which act on certain cellular materials have also been included. The information presented supports the view that the specific requirement of these organisms for sodium chloride and the effect of other chlorides and agents are to a great extent on a structural level and not based on osmotic protection only.

Methods

Halobacterium cutirubrum was used for all experiments. *Halobacterium salinarium* was used in one experiment with mixed salt solutions and the results were similar to those obtained with *H. cutirubrum*. Methods of growth and preparation of the cells were the same as described previously (1). Cells were grown in media containing 4.5 *M* sodium chloride and stock suspensions

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made in 4.5 *M* NaCl solutions. These suspensions were diluted 1:100 or 1:200 in 10-ml volumes of various concentrations of sodium, potassium, lithium, and ammonium chlorides, and changes in the optical density of the suspensions observed at intervals with a Coleman Junior spectrophotometer. In these preparations the concentration of NaCl added with the cells was 0.045 or 0.0225 *M*. With rubidium and cesium chlorides the stock suspension was prepared in 4.5 *M* NaCl solutions so that a 1:20 dilution gave the same optical density in 2-ml amounts as the other dilutions in 10-ml amounts. The final concentration of NaCl in these tubes was therefore 0.225 *M*.

Mixtures of chlorides were prepared by diluting appropriate volumes of concentrated solutions of the individual salts and the range is therefore limited by the individual solubilities.

Results

The Effect of Chlorides of Monovalent Cations on Turbidity of Suspensions and Morphology

The turbidity curves of cells suspended in decreasing concentrations of sodium, potassium, lithium, rubidium, cesium, and ammonium chlorides show striking variations (Fig. 1). Suspensions in NaCl alone show the preliminary increase in optical density reported previously (1). In all other chlorides there is a gradual, followed by a more definite, decrease in turbidity with decreasing concentration.

When these effects are correlated with the morphological forms of the cells

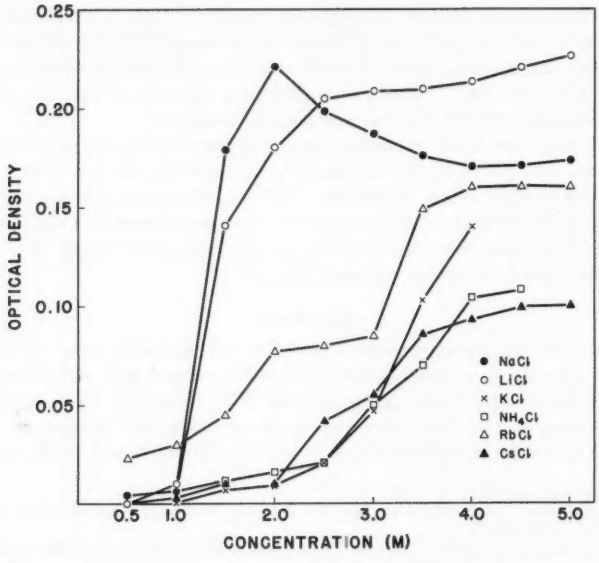

FIG. 1. Optical density of comparable suspensions of *H. cutirubrum* in decreasing concentrations of monovalent chlorides. For details see Methods section.

TABLE I

Morphology of *H. cutirubrum* after 3 hours in various concentrations of monovalent chlorides

SALT	CONCENTRATION, M									
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
NaCl	S	S	S	S1 T1	S2 T3	R1 T2	R	R	R	R
RbCl	G	G	G4 S1	G4 T1 S1	G6 T1	G3 T1	G1 T1	T	R3 T1	R
CsCl	–	–	S	S	S	S1 G1	S1 G1	R1 T3	R1 T1	R1 T1
KCl	–	–	–	S	S	T4 S1	T	R1 T3	X	X
LiCl	–	S	S	S	S	S	S	S	S	S
NH_4Cl	–	S	S	S	S	S	S	S	S	X

S = SPHERES. T = TRANSITION FORMS. R = RODS. G = GHOSTS. – = NO VISIBLE CELLS.
X = LIMIT OF SOLUBILITY. FIGURES INDICATE RELATIVE PROPORTIONS.

(Table I), it is clear that the optical properties of the suspensions are not the result of osmotic lysis alone and other effects must be considered.

If a drop of a concentrated solution of the various chlorides is applied to the edge of a wet mount of cells grown and suspended in 4.5 *M* NaCl, two distinct patterns of conversion from rods to spheres are observed. With KCl, RbCl, and CsCl the cells change via irregular transition forms, in the same way as in dilute solutions of NaCl (1). However, with these salts transition forms are found even in solutions as concentrated as 4.0 *M*. There is also a considerable decrease in cell numbers by lysis, even in the high concentrations of these other salts, as indicated by an increase in viscosity and of red pigment in the supernatant. In RbCl both transition forms and spheres may rupture, leaving behind ghosts, which do not disperse and must contribute to the turbidity. In CsCl the ghosts are mainly of the spherical forms and disperse similarly to ghosts in NaCl (1).

In the presence of LiCl and NH_4Cl there is an immediate change: two to five small spheres are formed from each rod and no intermediate forms have been observed. The increased optical density in 5.0 *M* LiCl is no doubt the result of greater scattering by the change in shape and the larger number of units. These lithium-produced spheres are quite resistant to change in osmotic pressure and there is no abrupt decrease in density until the concentration is reduced below 2.5 *M*. However, cells in NH_4Cl are much more fragile as indicated by the low optical density, even in 4.5 *M* NH_4Cl.

Morphological Changes in Mixtures of Chlorides

Lithium and potassium seem to exert quite different effects on the cells. The former seems to affect the structural integrity of the cell, while in the

TABLE II

Morphology and optical density of *H. cutirubrum* after 3 hours in mixtures of sodium and potassium chlorides (Order and size of letters give some indication of relative proportions; heavy line delimits concentration in which the rod form is maintained)

NaCl CONCENTRATION, M \ KCl CONCENTRATION, M	0.0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
0.0	–	–	S 7	S 8	S 10	S 25	TS 75	T 108	TR 120
0.5	S 3	S 10	ST 18	TS 28	TS 74	RT 105	R 122	R 137	
1.0	S 48	S 105	RTS 118	RT 145	R 148	R 142	R 130		
1.5	S 182	TRS 180	RT 150	RT 154	R 138	R 138			
2.0	ST 208	RTS 168	RT 152	R 147	R 140				
2.5	TS 194	RT 172	R 148	R 147					
3.0	RT 182	R 162	R 152						
3.5	R 168	R 152							
4.0	R 101								

R = RODS
T = TRANSITION FORMS
S = SPHERES
FIGURES = OPTICAL DENSITY

TABLE III

Morphology of *H. cutirubrum* after 3 hours in mixtures of sodium and lithium chloride (symbols as in Table II)

NaCl CONCENTRATION, M \ LiCl CONCENTRATION, M	0.0	0.5	1.0	1.5	2.0	2.5	3.0
0.0	–	S	S	S	S	S	S
1.5	S	S	ST	ST	S	S	S
2.0	ST	ST	TR	TR	ST	S	S
2.5	TS	TR	R	R	TR	TRS	
3.0	TR	R	R	R	R		

latter the cells disperse completely. It seemed worth while, therefore, to determine the effect of the sodium, lithium, and potassium chlorides in pairs on the morphology of *H. cutirubrum.*

In mixtures of NaCl–KCl the cells retain their rod shape as long as the total molarity of the mixture is greater than 3.5 (Table II). Transition forms appear at a total molarity of 3 and spheres at 2–2.5, much as in solutions of NaCl alone. It is also of interest that, as long as the molar concentration of sodium is 1 or more, the turbidity shows the characteristic increase with decrease in KCl concentration as has previously been noted only in NaCl solutions (1). Relatively small amounts of NaCl can therefore protect against the effect of KCl provided a high osmotic pressure is maintained. This indicates a specific action of sodium.

With LiCl–NaCl and LiCl–KCl the results are more difficult to interpret. In LiCl–NaCl (Table III) rod forms are found in a peak around a concentration of 1–1.5 *M* LiCl and above NaCl concentrations of 2.0 *M.* In LiCl–KCl mixtures (Table IV) it is difficult to tell because high concentrations cannot be obtained, but a similar distribution is indicated.

TABLE IV

Morphology of *H. cutirubrum* after 3 hours in mixture of potassium and lithium chlorides (symbols as in Table II; molar concentrations)

KCl \ LiCl	0.0	0.5	1.0	1.5	2.0	2.5	3.0
0.0	–	S	S	S	S	S	S
1.5	S	S	S	STR	Rt	TS	
2.0	S	S	TSR	R	RTS		
2.5	S	TR	Rt	R			
3.0	ST	R	R				
3.5	T	R					
4.0	TR						

Effect of Heat and Response of Heat-Treated Cells to Change in Salt Concentration

Rods, suspended in salt solutions of 3.0–5.0 *M* concentrations, may also be converted to spheres by increasing the temperature to 60–70° C. Microscopical examinations during the heating period show some transition forms, similar to those obtained by reducing the salt concentration, but the change to spheres by heat is usually rather sudden, and the cells retain their rod shape during most of the heating period.

The lower the salt concentration of the growth or suspending medium the shorter the time required to convert the rods to spheres by heat (Table V). Cells suspended in 4.5 *M* salt may be converted to spheres in about half the

TABLE V

Time in minutes required to convert rods to spherical forms by heating at 62° C. Cells grown in media containing 3.0 and 4.5 *M* NaCl and heated in various salt concentrations

Suspending medium, *M*	Salt concentration (*M*): 3.0	4.5
3.0	<1	2–4
3.5	2–4	4–5
4.0	8–10	13–15
4.5	11–14	20–22
5.0	24	41

time required for cells in 5.0 *M* salt, although no morphological difference can be detected either before or after the conversion.

The turbidity of suspensions immediately after conversion to spheres is only slightly less than that of the original suspension of rods. Direct counts show that the number of spheres is the same as the original number of rods and since there is no indication of breakage it would seem that one sphere is produced from each rod. On prolonged heating, however, the spheres rupture, the suspensions become viscous, and the turbidity decreases.

A nonviscous suspension of spheres was prepared by heating cells in 5.0 *M* salt at 60° C. When these spheres were suspended in decreasing salt concentrations, the suspensions showed only a slight increase in optical density at concentrations of 2.5 *M* compared with the original rods (Table VI). The density of suspensions of both rods and spheres decreased rapidly at concentrations below 2.0 *M*.

TABLE VI

Change in optical density of suspensions of rods and of heat-produced spheres of *H. cutirubrum* when suspended in different concentrations of NaCl*

	Salt concentration (*M*): 4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5
Rods	100	100	107	123	146	148	97	35	4
Spheres	100	97	98	100	106	103	78	28	2

*Rods grown in 4.5 *M* sodium chloride medium. Spheres produced from same cells by heating in 5.0 *M* salt at 60° C. Rods and spheres suspended in lower concentrations of NaCl, the turbidity read after 2 hours and expressed as percentage of optical density in 4.5 *M* salt.

Lysis of Spherical Forms in Various Chlorides and Mixtures of Chlorides

Suspensions of spheres were prepared in two ways: (1) by heating cells grown in 4.5 *M* salt in 4.0 *M* salt solution at 62° C, and (2) by suspending rods in a solution containing NaCl and LiCl at a concentration of 2.5 *M* each and adding 2.5 *M* LiCl dropwise until all the cells were converted to spheres. The spherical cells were collected by centrifugation at 3000 r.p.m. and washed once in 4.5 *M* NaCl.

These spheres were suspended in decreasing concentrations of the six monovalent chlorides. Those in KCl, RbCl, CsCl, and NH_4Cl lysed rapidly;

TABLE VII

Time required at 60° C to convert rods to spheres in solutions of NaCl and mixtures of NaCl with KCl or LiCl (Rods grown in media containing 5.0 *M* NaCl)

NaCl		NaCl 2.0 *M*		KCl 2.0 *M*		NaCl 3.0 *M*		NaCl 3.5 *M*		NaCl 4.0 *M*	
M	min	KCl, *M*	min	NaCl, *M*	min	LiCl, *M*	min	LiCl, *M*	min	LiCl, *M*	min
3.0	2	1.0	<1	1.0	<1						
3.5	5	1.5	2	1.5	4	0.5	2				
4.0	10	2.0	7	2.0	8	1.0	1	0.5	4		
4.5	25	2.5	21	2.5	24	1.5	<1	1.0	2	0.5	12
5.0	37					2.0	<1	1.5	<1	1.0	8

suspensions even in 4.0 and 5.0 *M* solutions were practically clear in a few minutes. The turbidity of comparable suspensions of spheres was higher in LiCl than in NaCl but in both salts the spheres began to lyse at concentrations below 2.0 *M*. Spheres produced by the two methods reacted similarly throughout.

When spheres were suspended in mixtures of NaCl–KCl and LiCl–KCl, both sodium and lithium protected them against the lytic action of the potassium salt.

When rods are heated in similar mixtures, the time required to convert rods to spheres in NaCl solutions or mixtures of NaCl–KCl is dependent on the total salt concentration of the solution (Table VII; cf. Table II). However, in LiCl–NaCl mixtures there is no relation to total concentration (cf. Table III) but the time increases as the NaCl concentration increases. The process is slowed down at lower temperatures; for example, it takes three times as long to produce spheres in the various LiCl–NaCl mixtures at 56° C as at 60° C.

The Effect of Urea on Cells

When cells are added to urea solutions, even as high as 10 *M*, they lyse immediately, indicating that it provides no osmotic protection. When urea is added to cells in sodium chloride solutions, the optical density decreases and the cells begin to lyse at higher salt concentrations as the concentration of urea is increased to 0.5 and 1.0 *M* (Fig. 2). At the same time the rods change, through transition forms to spheres. If the urea concentration is increased to 2 *M* the rods change to spheres even in 4.0 *M* NaCl and there is a considerable decrease in optical density. In the higher concentrations of urea the rods break up into four or five small spheres, similar to the change in LiCl or NH_4Cl. However there is not the increase in turbidity noted in LiCl, so presumably there is considerable lysis.

Spheres produced by heat or in LiCl–NaCl mixtures are lysed by urea similarly to the original rods.

Sodium chloride therefore has a protective effect against the action of urea but this effect is lost at concentrations much above 1.0 *M* urea.

Lysis of Cells by Surface-Active Agents

Since the changes noted above all seem to be related to the surface of the

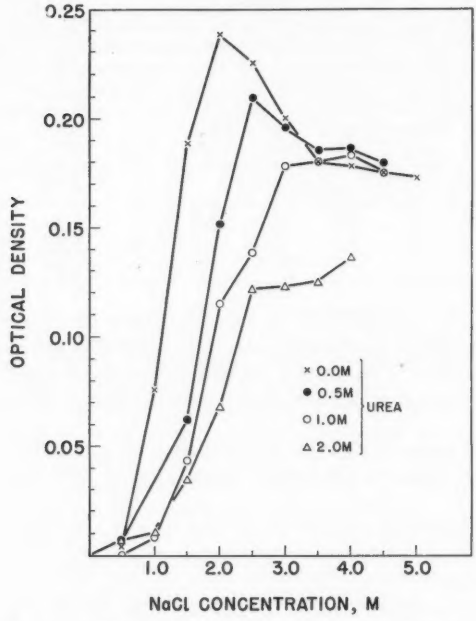

FIG. 2. Optical density of suspensions of *H. cutirubrum* in mixtures of urea and sodium chloride.

cell, the effect of several surface-active compounds was tested. The nonionic agents Tween 80 and polymyxin had no effect but two cationic compounds, cetyl pyridinium chloride and cetyltrimethyl ammonium bromide, and an anionic compound, sodium dodecyl sulphate, caused lysis. The lysis by these agents does not involve morphological changes and the cells disintegrate and disperse gradually.

Sodium chloride protects the cells from lysis and the rate of lysis increases with increasing NaCl but decreases with increasing LiCl concentration. However, NaCl has little protective effect on spheres formed in NaCl–LiCl mixtures. For example, rods suspended in 5.0 *M* NaCl have not lysed after 20 minutes in concentrations of 0.1 *M* cetyltrimethyl ammonium bromide but spheres have lysed after 2 minutes in concentrations of 0.0005 *M*.

Discussion

The results presented in this and a previous paper (1) indicate that sodium plays a unique role in maintaining the integrity of the cells of *Halobacterium cutirubrum*. However, concentrations of 3.5 *M* or more of NaCl are required to preserve the rod form of these cells. At lower concentrations the cell wall begins to lose its rigidity and irregular forms finally give way to spheres. Similar morphological changes are observed in solutions of RbCl, CsCl, and

KCl but here irregular transition forms are found in 4.5, 5.0, and 4.0 *M* concentrations respectively. In RbCl the spheres rupture as the concentration is reduced, leaving ghosts; in sodium, cesium, and potassium chlorides no ghosts can be found and it is not known whether the cells rupture as the result of an osmotic effect and the ghosts are of the same refractive index as the medium, or whether the sphere membranes are soluble in low salt concentrations.

These observations are consistent with an hypothesis that the cell walls of these organisms are held together rather loosely, as by hydrogen bonds, Coulomb forces, or "salt" linkages, and in the presence of high concentrations of NaCl the electrostatic forces are screened so that the bonds hold the organism in a rod shape. As the NaCl concentration is decreased, the electrostatic forces become operative and the bonding so weakened or stretched, at first in localized areas so that the cell bulges, and finally more generally so that it assumes a spherical form. This need not involve loss of cell-wall material. Heat would increase molecular movement, which would finally break the weak bonds and have an effect similar to reducing the salt concentration.

This hypothesis, however, does not take into consideration the specificity of sodium. The most obvious explanation would be that the hydrated sodium ion fitted the wall or membrane structure better than the larger lithium ion or smaller potassium ion. The potassium ion may be able to replace some sodium ions before weakening the wall structure sufficiently to be observed. Since hydrated rubidium, cesium, and ammonium ions are about the same size as potassium they might be expected to act in the same way. The experimental results indicate that rubidium and cesium are somewhat similar to potassium but the ammonium ion is quite different and resembles the much larger (ca. 2×) lithium ion.

As the effect of lithium and ammonium ions is quite different from that of the other ions, in that two or more protoplasts (spheroplasts?) are released per cell (as in some bacilli by lysozyme (4)), it is possible that these ions cause a more drastic disintegration of the cell wall. At present there is no other evidence of multicellularity in these organisms and surface tension may play a part. Although there is no great difference in the surface tension of solutions of sodium, potassium, lithium, and ammonium chlorides, if the latter two salts are responsible for a more complete removal of cell-wall material, surface tension phenomena may become operative.

The well-known effect of urea in breaking hydrogen bonds fits into this hypothesis, as well as the fact, reported earlier (1), that formalin, which increases cross-linkages, provides sufficient strength to the cell wall so that it can resist exposure even to water.

Surface-active agents act somewhat differently and the whole cell seems to disintegrate slowly. This may be the result of changes in the lipoproteins of the cell wall and membrane.

In all of these changes sodium chloride exerts a protective action which increases with its concentration, and the cell wall is more vulnerable to the "denaturing" effects of other monovalent chlorides, heat, etc., as the sodium chloride content of the medium is reduced.

Christian (3) showed that the moderate halophile *Vibrio costicolus* lysed in lower concentrations of sodium and lithium chlorides than of potassium or

ammonium chlorides and that *Halobacterium halobium*, a red-pigmented extreme halophile, was more sensitive to lysis in KCl than in NaCl. He attributed this solely to osmotic pressure. However, under these conditions, we think the effects on cell-wall structure are as important as the osmotic effects, and possibly more so.

Some of the effects of mixtures of salts are even more difficult to explain. With NaCl–KCl mixtures cells retain their form as long as the total molarity of the mixture is 3.5 or more and some sodium chloride remains in the mixture. This fits in with previous findings (2) that potassium can replace sodium in the growth medium of the red halophiles as long as the NaCl concentration is 1.5 *M* or more. The mixtures of NaCl and KCl which supported growth in the previous work (2, Table I) are amongst those in which all of the organisms are able to retain their rod form, but the organism can also retain its rod form in mixtures which do not support growth. It is evident that the specific requirement for sodium is related to physiological phenomena as well as to the structure of the cell wall.

No explanation can be offered at this time for the effect of LiCl in mixtures with NaCl or KCl. In NaCl–LiCl mixtures it seems that the cells retain their rod shape when the total molarity is 3.5 or more but only if the LiCl/NaCl ratio is less than 0.75. In KCl–LiCl mixtures the cells again retain their rod shape provided some lithium is present and the total molarity is 3.5 or greater. It also appears that a limit exists on the LiCl/KCl ratio but sufficient data are not available at present to be definite. Since cells cannot retain their rod shape in either concentrated LiCl or KCl it is difficult to understand why the addition of as little as 0.5 *M* LiCl to 3.5 *M* KCl prevents any change in morphology.

Although this work indicates that the sodium ion is of the greatest physiological importance to the red halophilic rods, its exact role in maintaining structure and growth is not known. It cannot be replaced by individual alkali ions, but the rod structure of these organisms can be maintained in the presence of some of these ions. However, such mixtures do not necessarily support growth, and considerably more information is required to understand the role and interaction of these ions in the metabolism of these halophilic organisms.

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IN VITRO SENSITIVITY OF LEPTOSPIRA TO VARIOUS ANTIMICROBIAL AGENTS[1]

J. G. COUSINEAU AND J. A. MCKIEL

Abstract

The *in vitro* sensitivity of *Leptospira icterohaemorrhagiae* was checked against 14 antimicrobial agents to find which might be added to leptospira culture media to control contamination without seriously depressing growth of leptospires. Estimates of the amounts of leptospira growth resulting in these modified media suggested that five agents were worthy of further study. The determination of leptospira population by microscopic counts showed that three of these compounds produced only slight to moderate inhibition. Two of these three antimicrobial compounds plus a fungicide were tested in combinations. Qualitative tests on these media were also carried out employing *L. ballum*, *L. canicola*, *L. pyrogenes*, and *L. sejroe*.

Based on results of these tests, a new selective medium for growing leptospires is suggested; it consists of a standard leptospira liquid medium to which are added 50 mg/l. sulfathiazole, 5 mg/l. neomycin sulphate, and 0.5 mg/l. actidione.

It is well known that contamination of cultures of pathogenic leptospires with other bacteria or fungi often results in overgrowth and destruction of the leptospires. Isolation of the etiologic agent in cases of leptospirosis is thereby rendered difficult. Contamination also tends to affect the accuracy of incidence values obtained in leptospira surveys of wildlife. The advantage of having available a selective medium for growing leptospires is, therefore, obvious. The only known media suggested for this purpose are those of Stavitsky (8), Stuart (9), and Dominguez (4). Stavitsky used sulfanilamide in a concentration of 4 g/l. of medium. Stuart found sulfaguanidine much more effective than either sulfanilamide or sulfadiazine. Dominguez observed that suppression of contamination is most effectively accomplished by addition of either dihydrostreptomycin at concentrations of 100–200 mg/ml or chloramphenicol at 200–500 mg/ml.

The study here reported was carried out to obtain information on the effects of a variety of antimicrobial agents on leptospires with a view to developing an improved selective medium.

The antimicrobial agents investigated included crystal violet, basic fuchsin, potassium tellurite, sodium azide, desoxycorticosterone, sulfaguanidine, sulfathiazole, furoxone, actidione,[2] bacitracin,[2] neomycin sulphate,[2] kanamycin sulphate,[3] and polymyxin B.[4]

Method

The serotypes used in this study were *Leptospira icterohaemorrhagiae*, *L. ballum* strain M-127, *L. canicola* strain Hond Utrecht, *L. pyrogenes* strain

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Contribution from the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario.
[2]Upjohn Co.
[3]Bristol Laboratories.
[4]Burroughs Wellcome Co.

Salinem, and *L. sejroe* strain Mallersdorf. The first four serotypes were obtained from Walter Reed Army Medical Centre, Washington, D.C. *L. sejroe* was obtained from the Animal Diseases Research Institute, Canada Department of Agriculture, Hull, Que. They were maintained in the laboratory by monthly passage in Korthof's medium plus rabbit serum (1). A stock solution of each of the agents to be tested was prepared and added to Korthof's medium to provide at least three concentrations for testing. Crystal violet was dissolved in 47% ethyl alcohol to a concentration of 1% and steamed for 10 minutes, as recommended by Salle (7). Basic fuchsin (0.03 g/l.) was dissolved in 10% ethyl alcohol. Desoxycorticosterone (0.025%) was dissolved in 1:1 ethanol–chloroform and then heated at 30° C overnight to remove the solvents (6). Other substances tested were dissolved in sterile Korthof's basic medium (peptone salt solution). These other substances were: sodium azide (50 mg per 100 ml), potassium tellurite (1% solution), actidione (1 g per 100 ml), sulfaguanidine (5 g/l.), sulfathiazole (5 g/l.). Antibiotic solutions were also prepared with this medium as the solvent.

Media with added test substances were dispensed in 5-cc amounts in 16-mm screw-cap tubes and incubated for 24 hours at 37° C as a sterility check. Three tubes were inoculated with 0.5 ml of a 5-day-old leptospira growth for each concentration tested in order to determine whether these concentrations would cause an inhibitory effect on *L. icterohaemorrhagiae*. Also one tube of standard Korthof's medium was inoculated as a control. The tubes were incubated at 29° C and checked daily for growth of leptospires by placing a loopful of culture on a glass slide and examining it by dark-field microscopy at 400× magnification. Growth was recorded as + (1–10 organisms per field), ++ (11–50 organisms per field), or +++ (more than 50 organisms per field). Each of the antimicrobial agents was tested at least twice against *L. icterohaemorrhagiae* according to the above method. The numbers of pluses recorded for all six tubes used in each test were averaged in order to express the result of each test as a single value for presentation in Tables II and III.

From this screening procedure, four compounds (sulfaguanidine, sulfathiazole, neomycin sulphate, and actidione) were selected for more critical evaluation using *L. icterohaemorrhagiae* as the test organism. Leptospira populations were determined by means of Chang's technique (2) employing calibrated pipettes to apply a known volume of formalized culture over a fixed area of a microscope slide. Duplicate preparations were made and, for each, 15 to 20 fields were counted daily for 4 days.

Combinations of sulfathiazole, neomycin sulphate, and actidione, at various concentrations, were also tested against *L. icterohaemorrhagiae*. Results were recorded qualitatively in terms of pluses and, in addition, population counts were made for two combinations, those with maximum and with minimum concentrations of the agents. A transfer was made after 72 hours to standard Korthof's medium to establish the leptospires' ability to multiply after having been grown in a medium with the antimicrobial agents.

To investigate possible differences between several serotypes in their reactions to these compounds, sulfaguanidine (1 g/l.), sulfathiazole (600 mg/l.), neomycin sulphate (8.5 mg/l.), and a combination of sulfathiazole

(50 mg/l.), neomycin sulphate (5 mg/l.), and actidione (0.5 mg/l.) were tested as well against *L. ballum*, *L. canicola*, *L. pyrogenes*, and *L. sejroe*.

Results

Agents Tested Singly

In the screen test, marked inhibition in the growth of *L. icterohaemorrhagiae* was caused by the following nine compounds (the concentration listed in brackets was the lowest of three tested): crystal violet (0.1 mg/l.), basic fuchsin (7.5 mg/l.), sodium azide (10 mg/l.), potassium tellurite (28.5 mg/l.), desoxycorticosterone (1.25 mg/l.), furoxone (25 mg/l.), chloramphenicol (25 mg/l.), polymyxin B (3 units/ml), and kanamycin sulphate (20 units/ml). Since these concentrations are lower than those employed to inhibit organisms most commonly encountered as contaminants of leptospira cultures, these compounds were not considered further in this study.

TABLE I

Concentrations at which certain agents commonly produce an antimicrobial effect

Antimicrobial agent	Concn.	Effective against:	References
Sulfaguanidine	125–200 mg/l.	Enterobacteriaceae	5
Sulfathiazole	50 mg/l.	Streptococci, staphylococci, salmonellae, Escherichieae	3
Actidione	0.1–1 mg/l.	Molds, fungi, Enterobacteriaceae, bacilli, streptococci, staphylococci	11
Neomycin sulphate	5–10 mg/l.	Enterobacteriaceae (except *E. coli*) bacilli, staphylococci	10
Bacitracin	5–10 units/ml	Bacilli, staphylococci, streptococci	10

The following five compounds were found not to inhibit growth of *L. icterohaemorrhagiae* when used at concentrations equal to or greater than those shown in Table I as being suitable for inhibiting contaminants: neomycin sulphate, bacitracin, sulfaguanidine, sulfathiazole, and actidione. In Table II it is seen that growth of *L. icterohaemorrhagiae* in standard Korthof's medium is comparable with growth of this organism in media containing sulfaguanidine (2 g/l.), sulfathiazole (600 mg/l.), actidione (1.5 mg/l.), neomycin sulphate (5 mg/l.), or bacitracin (15 units/ml). When higher concentrations of these compounds are used the rate of growth is seen to be reduced. For example, when sulfaguanidine, sulfathiazole, actidione, and neomycin sulphate were added at concentrations of 3 g/l., 1 g/l., 2 mg/l., and 8.5 mg/l. respectively, a +++ growth was attained only after 7 days of incubation. *L. icterohaemorrhagiae* populations exceeding ++ were never seen when concentrations

TABLE II

Growth of *Leptospira icterohaemorrhagiae* in modified Korthof's medium

Compound	Concn.	Growth after: 3 days	5 days	7 days
Sulfaguanidine	5 g/l.	++	++	++
	4 g/l.	++	++	++
	3 g/l.	++	++	+++
	2 g/l.	+++	+++	+++
Sulfathiazole	2 g/l.	++	+	+
	1 g/l.	++	++	+++
	600 mg/l.	+++	+++	+++
Actidione	2 mg/l.	++	++	+++
	1.5 mg/l.	+++	+++	+++
Neomycin sulphate	10 mg/l.	++	++	++
	8.5 mg/l.	++	++	+++
	6.75 mg/l.	++	+++	+++
	5 mg/l.	+++	+++	+++
Bacitracin	25 units	++	+	+
	15 units	+++	+++	+++
Control medium		+++	+++	+++

were 4 g/l. for sulfaguanidine and 10 mg/l. for neomycin sulphate. There was no growth after 3 days when sulfathiazole or bacitracin was added at a concentration of 2 g/l. or 25 units/cc respectively.

The results of the quantitative study on *L. icterohaemorrhagiae* growth determined in standard and modified Korthof's media are shown in Fig. 1. It may be seen that in media containing neomycin sulphate, only slight inhibition was produced by 5 and 6.75 mg/l., moderate inhibition by 8.5 mg/l., and marked inhibition by 10 mg/l. Sulfaguanidine at the concentrations tested caused marked inhibition of *L. icterohaemorrhagiae* while sulfathiazole at 600 mg/l. and bacitracin at 10 units/ml produced moderate inhibition.

Media containing neomycin sulphate (8.5 mg/l.), bacitracin (10 units/ml), sulfaguanidine, or sulfathiazole (600 mg/l.) supported growths of *L. ballum*, *L. canicola*, *L. pyrogenes*, and *L. sejroe* qualitatively similar to those obtained with *L. icterohaemorrhagiae*.

Growth of such contaminants as *Pseudomonas*, staphylococci, and others may be suppressed by neomycin sulphate at 7 mg/l., sulfathiazole at 50 mg/l., or bacitracin at 10 units/ml (Table I). Results given above indicate that incorporation of one or the other of these substances at these concentrations in a selective medium may be expected to produce from slight to moderate inhibition of growth of leptospires.

Agents Tested in Combination

Since a variety of microorganisms may be encountered as contaminants, it seems reasonable to expect that incorporation of more than one antimicrobial agent should provide a selective medium superior to those which

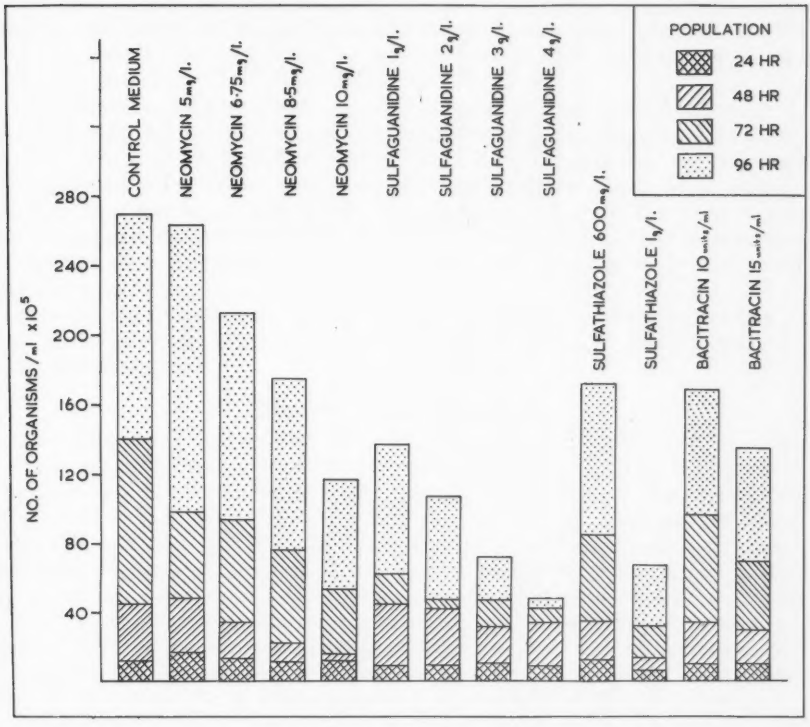

FIG. 1. *Leptospira icterohaemorrhagiae* growth in modified Korthof's media.

include only one of these agents. To this end, three of the antimicrobials used above were chosen for study when employed in combination.

Neomycin sulphate and bacitracin have similar antibacterial spectra. Of these two, neomycin sulphate was selected for further study because (*a*) it produced a lesser inhibitory effect on leptospiral growth when used at the concentration recommended in Table I, and (*b*) solutions of neomycin sulphate are stable for 1 year whereas solutions of bacitracin are stable for only 1 week. The remaining two agents included for study in combination were sulfathiazole to inhibit growth of *E. coli* and a few other organisms, and actidione to inhibit growth of fungi.

The results of tests on growth of *L. icterohaemorrhagiae* in media containing these three agents, each at two concentrations, are shown in Table III. Those concentrations to be tested were chosen according to the results obtained above in this study and those published by various authors (3, 10, 11). It may be seen that growth in media incorporating all eight combinations was similar to that in the control medium. During the third and fourth days however, growth was greater in media containing sulfathiazole at 50 mg/l.

TABLE III

Growth of *Leptospira icterohaemorrhagiae* in modified Korthof's medium with combination of antimicrobial agents

Antibiotic concentrations added to medium (mg/l.)			Population at times indicated (numbers shown in brackets are in millions)					Population in transfer medium at times indicated		
Sulfathiazole	Neomycin sulphate	Actidione	Day 1	Day 2	Day 3	Day 4	Day 7	Day 1	Day 2	Day 6
300	8.5	1	++(1.7)	++(3.7)	++(5)	++(4.8)	—(0)	+	+	—
300	8.5	0.5	++	++	++	++		+	+	—
50	8.5	0.5	++	++	+++	+++		+	+	+
50	5	0.5	++(1.6)	++(4.2)	+++(9.3)	+++(18.4)	+++(35)	++	++	+++
300	5	0.5	++	++	++	++		++	+	—
50	5	1	++	++	+++	+++		++	++	+++
300	5	1	++	++	++	++		+	+	+
50	8.5	1	++	++	+++	+++		+	+	+
Control medium			++(1.7)	++(4.7)	+++(10.1)	+++(27.7)	+++(42.5)	+++	+++	+++

regardless of the concentrations of neomycin sulphate and actidione. In media containing sulfathiazole at 300 mg/l. or neomycin sulphate at 8.5 mg/l., actively motile leptospires were seen but their ability to multiply was suppressed by these concentrations.

The efficacies of these media were evaluated further by subculturing to tubes containing control medium (without antimicrobial agents). The populations were recorded qualitatively for all tubes. In addition, the numbers of organisms per milliliter were determined in media in which the antimicrobials were at maximum and at minimum concentrations. On the basis of this test, the most satisfactory concentrations to be used in a selective medium appear to be sulfathiazole at 50 mg/l., neomycin sulphate at 5 mg/l., and actidione at 0.5 mg/l.

Once again, the other serotypes tested (*L. ballum*, *L. canicola*, *L. pyrogenes*, and *L. sejroe*) gave results qualitatively similar to those obtained with *L. icterohaemorrhagiae*.

Discussion and Summary

The isolation of leptospires is difficult and often impossible from cases of leptospirosis or in surveys to determine the incidence of these organisms if the inoculum is contaminated since bacteria may overgrow the leptospires. The purpose of this study was to find suitable antimicrobial agents which might be added to standard Korthof's medium to control growth of contaminants. To this end, the compounds used were added to Korthof's medium in increasing concentrations beginning at a level slightly lower than that commonly employed to inhibit growth of likely contaminants of leptospires. If the maximum concentration which did not seriously depress growth of the leptospires was found to be as high as or higher than that used to suppress growth of contaminants, that compound was selected for further study.

In the first step, 14 agents were screened including sulfaguanidine and chloramphenicol as recommended by Stuart (9) and Dominguez (4) respectively. Qualitative estimates of the amounts of leptospira growth resulting suggested that further examination of sulfaguanidine, sulfathiazole, neomycin sulphate, bacitracin, and actidione was justified.

In the second step, these five compounds which looked promising were checked by determining the resultant growth by microscopic counts as a confirmatory procedure. Following this test three agents were considered worthy of further study (sulfathiazole, neomycin sulphate, and actidione).

The third step consisted of studying the effect on leptospira growth of these three compounds added in combination: sulfathiazole for control of staphylococci, streptococci, and *E. coli*; neomycin sulphate for control of bacilli, *Pseudomonas* species, and staphylococci; and actidione for control of yeasts and fungi. The results of tests of the eight combinations of the three agents, each at two concentrations, indicated that the combination most suitable for suppressing growth of contaminants likely to be encountered in cultures of leptospires is sulfathiazole, 50 mg/l.; neomycin sulphate, 5 mg/l.; and actidione, 0.5 mg/l. It is planned to test this combination under field conditions in the near future.

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GROWTH INTERACTIONS OF ARTHROBACTER GLOBIFORMIS AND PSEUDOMONAS SP. IN RELATION TO THE RHIZOSPHERE EFFECT[1]

E. C. S. CHAN[2] AND H. KATZNELSON

Abstract

Studies on the influence of plant roots on the soil microflora have shown that the number of Gram-negative rods in the rhizosphere is relatively higher than the number of Gram-positive rods, coccoid rods, and sporeforming types. This ecological phenomenon was duplicated and studied in a model system using a *Pseudomonas* sp. as representative of the rhizosphere and *Arthrobacter globiformis* as representative of the soil flora. Growth of *A. globiformis* was strongly suppressed in the presence of the pseudomonad in root extracts of mature plants and in a medium containing casamino acids, yeast extract, glucose, and mineral salts (CAYG medium); suppression was less marked in soil extract. The pseudomonad was unaffected in the association. *A. globiformis* was inhibited in the first 48-hour incubation in CAYG medium by acid elaborated by the pseudomonad; the pH dropped to 5.3 in 16 hours but rose steadily to alkaline conditions after 48 hours, resulting in a delayed increase in the number of *A. globiformis* to approximately that of a pure culture, in 5 days. Under cultural conditions favoring pigment production by the pseudomonad, growth of *A. globiformis* was completely inhibited throughout this period. Another toxic principle was also produced by the pseudomonad. This substance was biologically active in mixed culture against the *Arthrobacter* but was present in low concentration.

Introduction

The ultimate problem in microbiology, according to Woods (9), is to explain in biochemical terms the life of microbes in the mixed association in which they exist in nature. Natural habitats are difficult to analyze because of the environmental variables and the heterogeneity of microbial species. Some simplification of experimental design is therefore necessary. One convenient and often valuable approach is that of a model system using mixtures of known cultures in the laboratory. Its advantage lies in making possible the analysis and synthesis of interactions under controlled conditions. In this sense, a microbial laboratory model is a useful tool in ecological studies to delineate the well-known phenomena of antibiosis, symbiosis, synergism, and commensalism.

The microbial flora of soils and rhizospheres has been extensively surveyed (1, 3, 5, 7, 8). These studies have shown that in the rhizosphere Gram-negative rods (represented by *Pseudomonas* species) are relatively more numerous than Gram-positive rods, coccoid rods, and sporeforming types (such as those belonging to the genera *Arthrobacter*, *Nocardia*, and *Bacillus*). In root-free soil, the latter groups predominate over the Gram-negative rods. The purpose of this investigation was to study the interaction of a representative rhizosphere organism and one from root-free soil in order to provide a rationale for the ecological observations noted.

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Contribution No. 520, from the Microbiology Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Canada.
[2]National Research Council Postdoctorate Fellow, in collaboration with the Canada Department of Agriculture, 1960–62.

Materials and Methods

Cultures

The organisms used were a *Pseudomonas* sp. isolated from the rhizosphere of wheat and *Arthrobacter globiformis* 425 (from the culture collection of the Microbiology Research Institute), a typical representative of the bacterial flora of the soil itself. Both organisms were able to develop together on prolonged incubation on crowded agar plates. The two species were easily differentiated by colonial characteristics (Fig. 1).

Culture Media

The media employed were root extracts, soil extract, and a complex medium prepared with dehydrated ingredients.

(*a*) Root extracts were prepared from mature soybean and barley plants. Roots were washed free of soil in running water and air-dried for about 12 hours. Eighteen grams of dried roots was soaked in 350 ml distilled water for 3½ hours with occasional agitation, and the solution filtered through Whatman No. 12 paper. About 240 ml of extract (pH 6.4) was recovered. The extract was neutralized and then sterilized by autoclaving at 15 lb for 15 minutes.

(*b*) Soil extracts were prepared by adding 500 g of garden soil to 1 l. tap water and heating in an autoclave for 30–40 minutes at 15-lb pressure. The slurry was then filtered through Whatman No. 1 filter paper and the filtrate brought up to a final volume of 1 l. with tap water. The extract was autoclaved at 15 lb for 15 minutes (final pH, 6.2).

(*c*) The complex medium employed (CAYG) was of the following composition: casamino acids, 5 g; yeast extract (Nutritional Biochemicals Corp.), 5 g; glucose, 5 g; salt solution, 1 l., pH 6.8. The salt solution was made by adding to 900 ml of distilled water 10 ml of each of the stock salt solutions prepared by dissolving each of the following salts in 100 ml of distilled water: K_2HPO_4, 10 g; KNO_3, 5 g; $MgSO_4.7H_2O$, 2 g; $CaCl_2$, 1 g; NaCl, 1 g; $FeCl_3.6H_2O$, 0.1 g. The solution was adjusted to pH 7.0, heated to boiling, cooled, and filtered through Whatman No. 12 filter paper. The volume was adjusted to 1 l. for use in medium preparation.

For some experiments the medium was modified by reducing the glucose content to 0.1 and 0.01% (designated as $CAYG_{0.1}$ and $CAYG_{0.01}$, respectively).

Preparation of Inoculum

Inoculum was prepared by growing the organisms on slants of Difco Antibiotic Medium 2 (Bacto-Penassay Base Agar) for 24 hours at 25° C. Cells were transferred to fluid medium and the turbidity adjusted to a Klett reading of 51 using a red filter (No. 66). Of this suspension, 0.01 ml was used to inoculate 20 ml of medium.

Growth and pH Measurements

Growth of the pure and mixed cultures was followed by means of plate counts on Difco Antibiotic Medium 2. Pigments were not produced by the pseudomonad in this substrate. Serial dilutions were prepared by using Difco Antibiotic Medium 3 (Bacto-Penassay Broth) as the diluent since

PLATE I

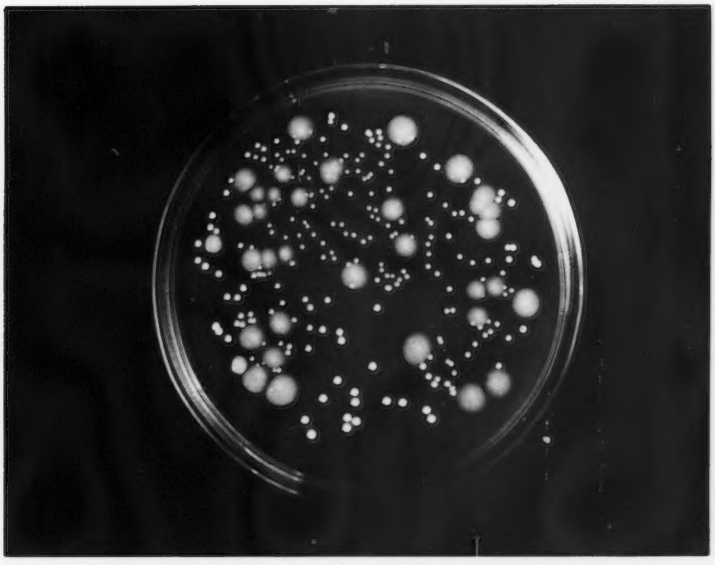


FIG. 1. Colonial morphology of *Pseudomonas* sp. and *Arthrobacter globiformis*. The *Arthrobacter* colonies are smaller and can even develop on top of the *Pseudomonas* colonies on prolonged incubation.

preliminary experiments showed a rapid loss of viability of *A. globiformis* in distilled water. All pH values were determined with a Beckman pH meter. Plates and liquid cultures were incubated at 25 ± 2° C; the latter were shaken at about 110 revolutions per minute on a rotary shaker.

Filtrate Preparation

Filtrates of the pseudomonad and of the mixed cultures were prepared by centrifugation of the cells at 12,100 *g* for 20 minutes in a Serval Superspeed centrifuge. Sterility was achieved by subsequent filtration through ultra-fine sintered-glass filters. Unless otherwise specified, all filtrates were adjusted to pH 7.2 before use.

Results

Model System with Root and Soil Extracts and Medium CAYG

When the pseudomonad and *A. globiformis* were grown together in mature soybean root extract, the latter organism was inhibited as shown in Fig. 2, whereas the former was unaffected. The same interaction pattern was also obtained with extracts of mature barley root. Pigment production by the pseudomonad did not occur in these extracts.

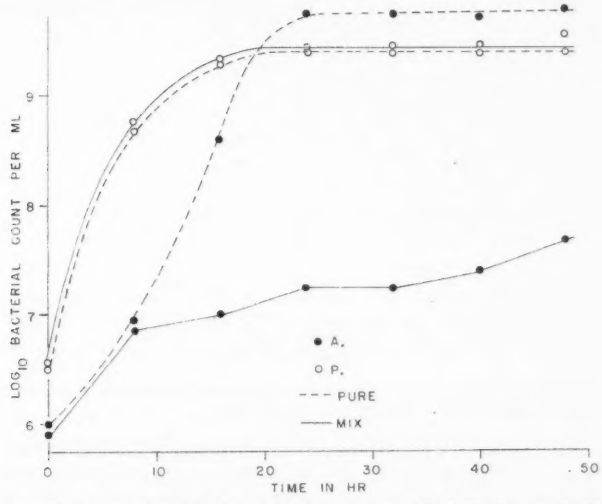


FIG. 2. Growth interaction of *Pseudomonas* sp. (P.) and *Arthrobacter globiformis* (A.) in mature soybean root extract.

Growth of a mixed culture of the two organisms in soil extract also proved unfavorable for *A. globiformis*; the pseudomonad was also non-pigmented in soil extract. The same general pattern of suppression of the *Arthrobacter* was observed as in Fig. 2 but this inhibition was very much less pronounced than in root extract. At the time of maximum inhibition (16 hours) the ratio of the number of pseudomonad to the number of *A. globiformis* in root extract of soybean was 213:1 whereas the ratio in soil extract was 4:1.

In CAYG medium, the *Arthrobacter* was also inhibited in mixed culture (Fig. 3) whereas the pseudomonad was unaffected and produced no pigment. The ratio of *Pseudomonas* to *Arthrobacter* was 9875:1. It may also be seen that the *Pseudomonas* has not or only a very short lag phase whereas the lag period of *A. globiformis* in pure culture is approximately 4 hours. In parallel studies it was found that the generation time of the pseudomonad in this medium is about 48 minutes while that for *A. globiformis* is about 84 minutes.

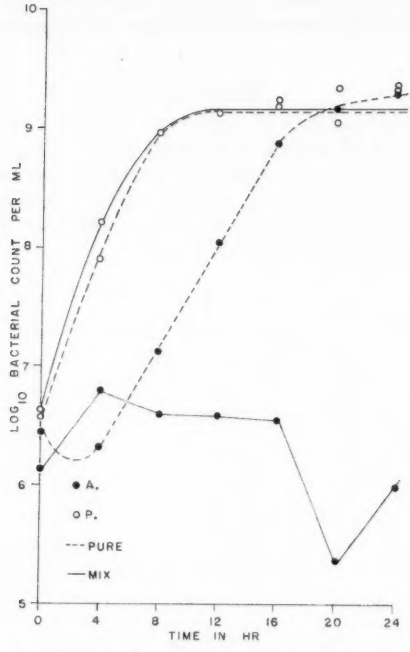
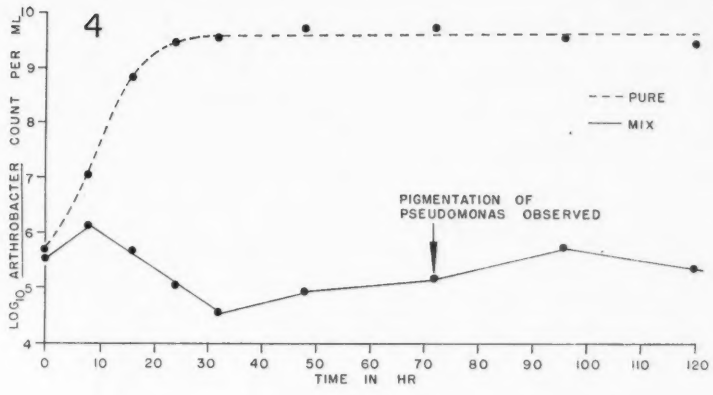


FIG. 3. Growth interaction of *Pseudomonas* sp. and *Arthrobacter globiformis* in a casamino acid – yeast extract – glucose – salts medium.

The effect of incubating a mixed culture in medium CAYG for an extended period is shown in Figs. 4 and 5. If Difco Bacto-yeast extract (Lot 437697) is used instead of N.B.C. yeast extract, the formation of a green fluorescent pigment by the *Pseudomonas* sp. is favored. (The routine use of N.B.C. yeast extract, Lot 7590, obviated this complication.) From Fig. 4 it can be seen that under these conditions growth of *A. globiformis* is suppressed throughout the 5 days of incubation, with no recovery. However, if pigment is not produced, counts of the *Arthrobacter* begin to increase on or about the second day so that its population finally approaches the level of the pure culture (Fig. 5).

The question remained concerning the factor(s) responsible for inhibition of *A. globiformis* in the absence of or before pigment formation. The possi-



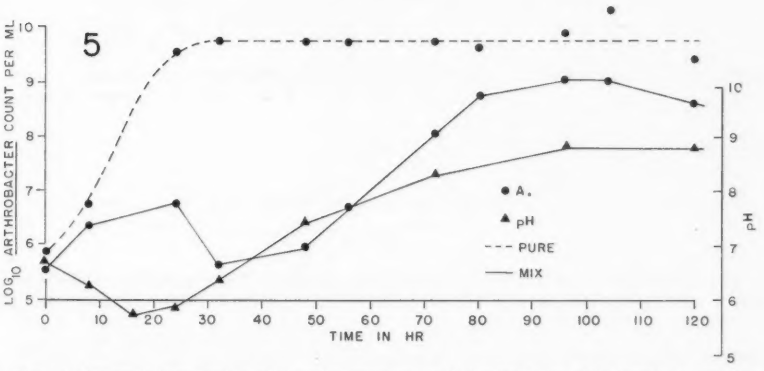


FIG. 4. Growth interaction of *Pseudomonas* sp. and *Arthrobacter globiformis* in medium CAYG in the presence of the green fluorescent pigment of the pseudomonad.
FIG. 5. Growth interaction of *Pseudomonas* sp. and *Arthrobacter globiformis* in medium CAYG in the absence of pseudomonad pigmentation.

bility existed that the production of acid by the pseudomonad in the presence of a carbohydrate might inhibit growth of the *Arthrobacter*. Therefore, pH determinations were made of a growing mixed culture at different times and the results obtained are also shown in Fig. 5. The growth curve of *A. globiformis* in mixed culture follows roughly the pH curve and suggests inhibition of *A. globiformis* by the pseudomonad as a result of acid production since a pure culture of the *Arthrobacter* becomes alkaline in this medium. Further experiments indicated that growth of *A. globiformis* is retarded when the initial pH is about 5.5; initial pH values from 6 to 9 do not affect the rate of growth. This is supporting evidence for the inhibition of this organism by acidity since the pH of the mixed culture may decrease to 5.3 in 16 hours. As final evidence of the pH effect, it was shown that the *Arthrobacter* is suppressed in a 16-hour unadjusted culture filtrate of the pseudomonad (pH 5.3). Raising the pH to 6.8 resulted in normal growth (Fig. 6).

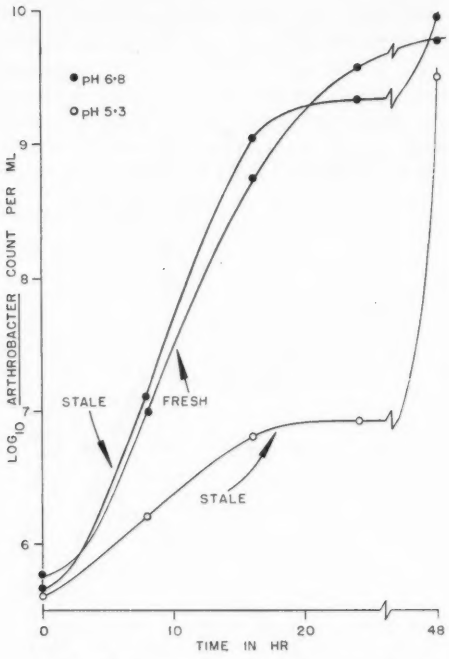


FIG. 6. Inhibition of *Arthrobacter globiformis* by the acid elaborated by *Pseudomonas* sp. *A. globiformis* was inoculated into sterile 16-hour pseudomonad filtrates with pH unadjusted, or adjusted to 6.8.

Additional Inhibitory Effects

In further experiments on the pH effect it was found that the addition of powdered $CaCO_3$ (0.1%) to a mixed culture raised the pH above 6.0, yet *A. globiformis* was still suppressed (Fig. 7). Furthermore, when the glucose in CAYG medium was reduced from 0.5% to 0.1 and 0.01%, the pH of a mixed culture did not drop below 6.5; nevertheless, as may be noted in Fig. 8, inhibition of *A. globiformis* was still marked. These results suggested the presence of other inhibitory factors in the system. Accordingly, growth of *A. globiformis* was followed in filtrates of the pure *Pseudomonas* culture and of mixed cultures using $CAYG_{0.01}$ broth. Inhibition occurred with 24-hour but not with 16-hour filtrates and indicated that the pseudomonad was again responsible for the effect. Table I shows the growth of *A. globiformis* in a 24-hour *Pseudomonas* culture filtrate of $CAYG_{0.01}$ broth. Dilution with distilled water resulted in increased growth of the organism, suggesting that a nutritional deficiency was not induced by the pseudomonad. This evidence further supports the hypothesis that an inhibitor is produced by the pseudomonad but only in low concentration at 24 hours, since slight dilution with fresh medium (or with water) overcomes the effect.

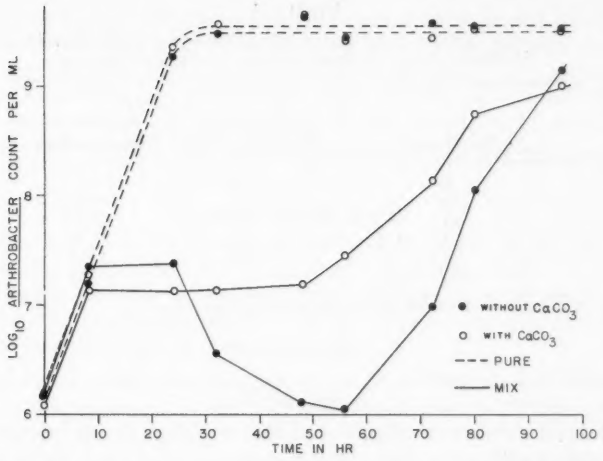


FIG. 7. Effect of calcium carbonate on the growth of *Arthrobacter globiformis* in mixed culture in medium CAYG.

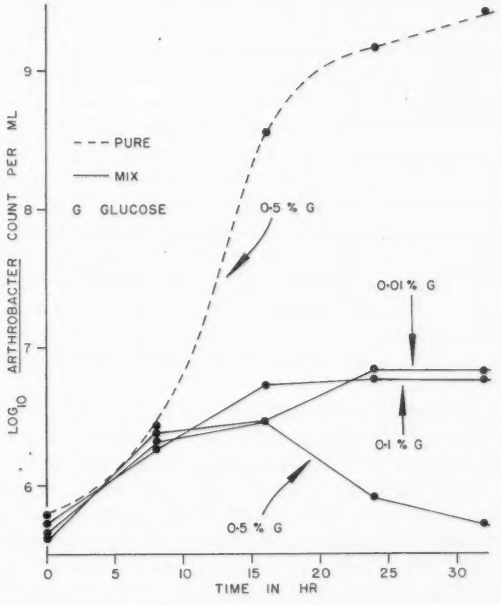


FIG. 8. Effect of reduced glucose in medium CAYG on the mixed culture growth of *Arthrobacter globiformis*.

TABLE I

Effect of dilution upon the growth of *Arthrobacter globiformis* in filtrate from a 24-hour culture of a *Pseudomonas* sp.

% Diluent	Diluent: Distilled water	Diluent: Fresh medium
0	37*	37
20	147	490
40	250	910
60	210	1740
80	440	1560
100	—	530

**A. globiformis* in millions per ml (after 24-hour incubation).

Discussion

The model system used in this investigation was designed to by-pass the difficulties inherent in any population analysis of a complex natural habitat. Although there are obvious dangers in such simplification, the results obtained with the mixed cultures reflect in some measure the microbiological events at the surface of a plant root, that is, the development of a bacterial flora in which Gram-negative rod-forming organisms predominate over Gram-positive rods, coccoid rods, and sporeforming types. In addition, the results provide a physiological basis for the observed shift in the microbial equilibrium of the soil as a result of penetration by a growing root. Population studies, as recorded in earlier publications (2, 4, 11), have all shown that the bacterial flora of the rhizosphere is more active metabolically and grows more rapidly than that of the root-free soil. The experiments reported herein not only yield further support for this but also provide additional possible reasons for the predominance of Gram-negative organisms as represented by *Pseudomonas* sp. in the root zone—acid production, pigment production, and liberation of inhibitory substances. It was significant that the inhibition of *Arthrobacter* in mixed culture in root extract was so much greater than in soil extract. The nutritional level of the latter in respect to amino nitrogen and sugars is very low and under such conditions the *Pseudomonas* does not develop normally, a situation which obtains in soil. The reverse effect must occur at the root surface or, as in the present experiments, in root extract, where sufficient nutrients are available to permit the *Pseudomonas* to grow rapidly.

The observation that only 24-hour and not 16-hour filtrates of pure *Pseudomonas* cultures (as well as mixed cultures) inhibited the *Arthrobacter* under conditions which precluded acid production is of interest in that it suggests the formation of still other inhibitory substances. Yet in mixed culture in the absence of acid production (Figs. 7 and 8) the *Arthrobacter* was inhibited as early as 8 hours after inoculation. A possible interpretation of this apparent discrepancy is that the inhibitory material was either of very low toxicity or was produced in such small amounts as not to be detectable in the culture filtrate before 24 hours. The early inhibition in mixed culture could be due to the intimate contact of the two organisms, which would permit one to affect

the other even though only minute amounts of toxic material were produced. Such a phenomenon may be effective also in natural habitats, such as soil, in which readily detectable amounts of antibiotic may not be formed (10). Antibiotic effects may nevertheless occur in various loci—in soil crumbs, on pieces of organic material—in which the intimate contact of microorganisms would render even extremely small amounts of inhibitory substances highly effective (6).

Acknowledgment

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PRIMARY OXIDATION OF SOME ORGANIC COMPOUNDS IN REMOISTENED AIR-DRIED SOIL[1]

J. DROBNÍK

Abstract

The effect of adding citrate, caproate, acetate, lactate, alanine, or glutamate on the respiration curves obtained from remoistened soil was studied. The curves can be analyzed as proposed in our experiments with glucose. The curves with caproate and acetate are different from the basic type described with glucose; these two acids inhibited the endogenous respiration. The amount of oxygen consumed during primary oxidation was approximately one-half of the amount necessary for complete oxidation with all substrates studied. Glutamate was most favorable for enzyme synthesis. The oxidation of alanine was very similar to that of lactate. Further study of respiration curves, especially those of acetate and caproate, might be useful in elucidation of soil oxidations.

Introduction

In our previous papers (2, 3, 4) we proposed an analysis of respiration curves of a soil sample treated with glucose. We studied the cumulative curve of oxygen consumption and also the derived curve which showed the course of the respiration rate. The main parameters of these curves are as follows: (1) the amount of oxygen consumed during a primary oxidation, (2) the relative magnitudes of oxidative and assimilative components, (3) the form of the logarithmic increase of the respiration rate during the assimilative part.

We deduced from these parameters some features of the biological component of the soil samples:

(1) From the amount of oxygen absorbed per molecule of the added substrate we deduced what part of the carbon supply was oxidized during the primary oxidation and what part was assimilated to be left in the soil as a carbon source for humus synthesis.

(2) The size of the oxidative component indicated by the initial respiration rate shows the magnitude of the oxidizing enzymatic system originally present in the soil sample (in living cells, or adsorbed on the soil particles), i.e., it is a measure of an actual activity of the microflora.

(3) The course of the logarithmic increase of the respiration rate during the assimilative phase shows the period of latency preceding the elaboration of new enzymes and the rate of this elaboration. These data characterize the potential activity of the microflora.

In all our experiments we used glucose as the substrate. Glucose is readily oxidized by almost all heterotrophic soil microorganisms. Its degree of oxidation, designated ω (2), equals 4, i.e., four electrons are necessary for each carbon atom to bring it to a completely reduced form. The object of the present paper is to ascertain whether the method of analysis previously described can be used for substrates differing from glucose in three ways: (*a*) they are not so readily oxidized by all microorganisms, (*b*) their degree of oxidation is

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Contribution from the Laboratory of Biophysics, Faculty of Sciences, Charles University, Prague, Czechoslovakia.

higher or lower than 4, and (*c*) their chain is shorter. As examples of such substrates the following were selected for study: citric acid and caproic acid, representing six-carbon compounds with different degrees of oxidation, $\omega = 5$ for the former and $\omega = 2.7$ for the latter; acetic and lactic acids, representing substrates with the same degrees of oxidation as glucose but with shorter molecules; alanine, representing a nitrogen-containing molecule similar to lactic acid; and, finally, glutamic acid representing an amino acid which plays a most important role in nitrogen metabolism of microorganisms.

Only the assimilative part of primary oxidation was studied in the present work, the oxidative part being destroyed by drying.

Materials and Methods

The same meadow sandy-loam reported in our previous papers (3, 4) was air-dried immediately after sampling and stored at 4° C; no changes in respiration were observed during the series of experiments.

Four grams of soil was weighed into a conventional Warburg vessel (area of bottom 11–12 cm²). No difference in respiration was observed using 0.9, 1.0, and 1.1 ml of water; therefore 1.0 ml of substrate solution or water was used. The pH of all substrate solutions was adjusted to 6.0 using 1 *M* KOH, and they were added directly to the soil at zero time. The vessels were incubated at 28° C without shaking. The first reading was made after 1 hour. All results are the mean values of at least four replicate measurements. Statistical treatment was done as follows: From measured values $x_1, x_2, \ldots, x_n$ the mean value $\bar{x}$ was determined. The standard deviation s_x and standard error $s_{\bar{x}}$ were calculated in the usual way and, using the t test, the necessary difference (at 5% level of significance) was established, $t_4 = 3.182$ or $t_5 = 2.776$, etc. From the values $\bar{x} \pm ts_{\bar{x}}$ calculated for endogenous respiration and from $\bar{x}' \pm ts_{\bar{x}'}$ for total respiration with substrate, the net respiration value for substrate with its necessary difference was calculated as follows:

$$(\bar{x}' - \bar{x}) \pm \sqrt{(ts_{\bar{x}'})^2 + (ts_x)^2}.$$

The fiducial limits are presented graphically in the figures for endogenous respiration and oxidation of acetate. In other cases the level of significance is reported in the text.

Results

The variation of the endogenous respiration rate with time is presented in Fig. 1. It has the same single-peak shape reported by Douglas and Tedrow (1) when they used soil that had been stored air-dry. The peak occurred between the 9th and 15th hours. This endogenous respiration rate has been subtracted from all further data. The endogenous respiration curve was determined four times (always four replicate samples) during the whole period of the experiments. A certain variability, observed in the initial portion of the curve before the maximum was reached, was caused by fluctuation in the time at which the maximum occurred.

Citrate oxidation produced relatively simple curves, which are presented in Fig. 2. On the other hand, the oxidation of caproate, which represents the second six-carbon compound, yielded complex curves which appear in Fig. 3.

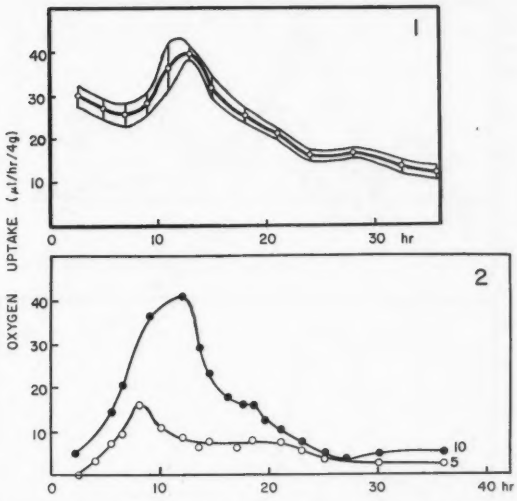

FIG. 1. Endogenous respiration rate of 4 g of remoistened air-dried soil. The limits of significance (5% level) are given as $\pm sz.t.$

FIG 2. The respiration rates of a soil sample with 5 and 10 μM of citrate.

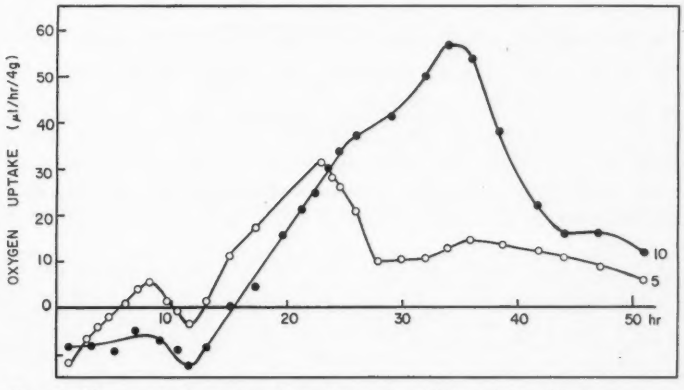

FIG. 3. The respiration rates of a soil sample with 5 and 10 μM of caproate.

The distinctive shape of the curves for both concentrations of caproate is significant with the exception of the fluctuations up to the 15th hour observed on the curve of higher concentration. The peak at the 8th hour on the low-concentration curve was significant and was confirmed by several repetitions.

The results obtained with acetate as substrate produced unusually complex curves. The average respiration values with their necessary differences are given graphically in Fig. 4. The first minimum of both curves, shown here at

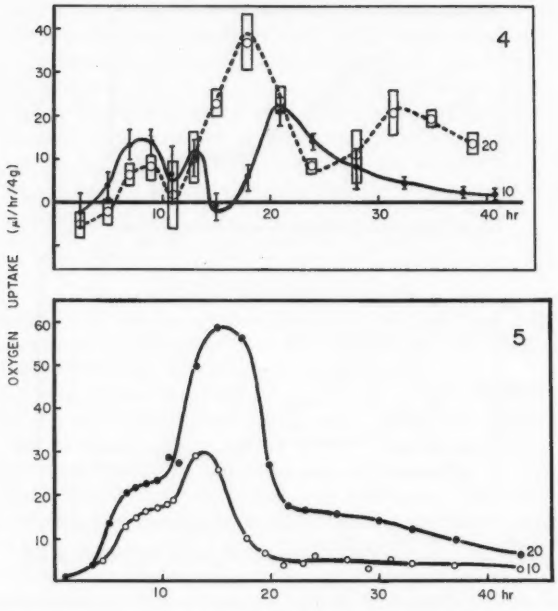

FIG. 4. The respiration rates of a soil sample with 10 and 20 μM of acetate. Significance limits as in Fig. 1.

FIG. 5. The respiration rates of a soil sample with 10 and 20 μM of lactate.

the 11th hour, while not statistically significant, was confirmed by four replicate experiments. However the replications differed slightly in the time and magnitude of the minimum. Also the great variability at 18 and 21.5 hours was caused by fluctuation in the time when the curve started to rise. These examples demonstrate one disadvantage of using the form of statistical analysis on such curves.

The oxidation of lactate is given in Fig. 5. The delay between the 6th and 10th hours is significant. The oxidation curve of alanine (Fig. 6) is very similar to that of lactate. Four concentrations of glutamic acid were used, and corresponding curves are shown in Fig. 7. The oxidation data for all substrates are summarized in Table I.

Discussion

From the results it is apparent that primary oxidation is as well pronounced with these substrates as was shown earlier for glucose (3). The shapes of the respiration curves are often characteristic and their detailed study will likely provide further information about the nature of oxidation processes in the soil.

In the experimental conditions described in the present paper the proportion of the substrates oxidized during primary oxidation is about the same for

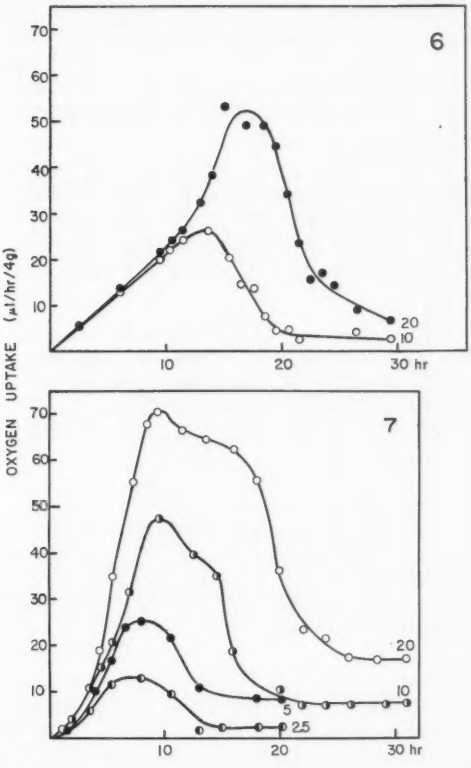

FIG. 6. The respiration rates of a soil sample with 10 and 20 μM of alanine.
FIG. 7. The respiration rates of a soil sample with 2.5, 5, 10, and 20 μM of glutamate.

each (see final column, Table I). The error using smaller concentrations is relatively high. The results with greater concentrations of substrates do not show any significant difference, with the exception of the greatest concentration of glutamate. All substrates are approximately 47% oxidized. This value is much higher than those obtained for glucose in the previous papers, namely 24.5% and 27.6%. We suppose that glucose is the best substrate for assimilation and therefore its assimilation is much greater than its oxidation.

Although the values summarized are roughly uniform, the shape of the respiration curves is very characteristic. Those for citrate, alanine, and glutamate have the simple basic form as previously described for glucose, differing only in the logarithmic rise of respiration rate; glutamate stimulated the respiration most of all. The logarithmic rise is characterized by an equation:

$$Q_{O_2} = 2^{(1/1.07)t}.$$

Corresponding expressions for respiration rate for citrate and alanine are:

TABLE I

Relative oxidation of substrates during primary oxidation calculated from oxygen consumption*

Substrate	Concn., μM	Max. resp. c,† hours	Prim. oxid. d,‡ hours	Oxygen uptake (at time d)			
				Endogenous, μl	Substrate, net, μl	Mole ratio O_2/substr. M/M	Relative oxidation, %
Citrate	5	9	16	498.0±23.3	187.4±41.4	1.68±0.093	37.4± 8.2
	10	12	25	688.1±31.8	474.7±51.1	2.12±0.218	47.4± 5.1
Caproate	5	23	28	738.4±34.7	268.8±59.2	2.40±0.133	30.0± 6.6
	10	34	44	934.2±36.1	810.0±39.9	3.61±0.178	45.1± 2.2
Lactate	10	14	20.5	609.5±27.9	275.8±44.2	1.23±0.197	41.0± 6.6
	20	16	22	641.5±29.2	621.1±46.0	1.39±0.205	46.2± 3.4
Alanine	10	13	21	620.2±28.3	295.9±48.3	1.32±0.215	44.0± 7.2
	20	17	24	672.6±31.0	620.0±56.6	1.38±0.253	46.1± 4.2
Glutamate	2.5	7	13	394.8±20.3	104.2±31.1	1.86±0.550	41.7±12.4
	5	8	14	434.4±21.3	225.8±40.6	2.01±0.360	45.2± 8.1
	10	9.5	22	641.5±29.2	487.6±34.8	2.17±0.156	48.8± 3.5
	20	9.5	26	705.1±32.6	1037.0±58.5	2.31±0.131	51.8± 2.9

*Data are given as mean value ± necessary difference at 5% point.
†The time of maximum respiration rate.
‡The time of the end of primary oxidation.

$Q_{O_2} = 2.14 \times 2^{(1/2.02)t}$ and $Q_{O_2} = 5.63 \times 2^{(1/4.94)t}$, respectively, i.e., the "mean doubling time" is approximately twofold longer for the former and fivefold longer for the latter.

The delay in the respiration for lactate and the form of the respiration curves for acetate and caproate cannot as yet be explained. Only the second peak in the respiration for acetate is dependent on the substrate concentration. This unusual shape occurred only with soil that had undergone air-drying. The respiration curves obtained when the soil had not been air-dried gave a simple form whether acetate, caproate, or the other substrates were used.

An interesting finding was the depression of oxygen consumption for the first 4 hours when acetate or caproate was added to remoistened soil, as shown in Table II.

TABLE II

Effect of acetate and caproate on the reactive oxidation of air-dried and remoistened soil

Substrate added	Respiration by soil sample in μl O_2 per hour per 4 g*		
	Maintained moist	Air-dried and remoistened	Reactive oxidation†
None	21.92±1.72	30.09±2.45	8.17
Acetate, 20 μM	30.00±2.67	25.19±1.43	3.27
Caproate, 5 μM	38.00±0.47	26.65±1.68	4.73

*The respiration rate is calculated from the first 4 hours. The mean values and necessary difference at 5% point are given.

†I.e., the difference between the endogenous respiration of air-dried and remoistened soil and the endogenous respiration of soil which had not been air-dried. The substrates which had been added to air-dried and remoistened soil were not oxidized during the first 4 hours; therefore the respiration in this interval can be compared with the endogenous respiration of the moist sample.

The air-drying and remoistening stimulated the endogenous respiration by about 36%. We call this addition "reactive oxidation". When acetate and caproate are added to soil treated in this way this reactive oxidation was suppressed to about 40% and 58%, respectively, of that occurring in soil without any substrate. This peculiar effect of these compounds might be used to study the nature of reactive oxidation.

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THE EFFECT OF FATTY ACIDS ON GROWTH AND ANTIBIOTIC PRODUCTION BY STREPTOMYCES[1]

JEROME J. PERRY[2]

Abstract

The ability of lipid materials to enhance the growth of *Streptomyces* has been demonstrated. The addition of corn oil or unsaturated fatty acids not only increases the total growth in the presence of carbohydrate but also provides a readily utilizable source of carbon and energy in the absence of carbohydrate. For most antibiotic-producing cultures investigated, the antibiotic produced was enhanced in direct proportion to the increase in growth. Organisms producing Cycloheximide or polyene-type antibiotics were disproportionately stimulated in the production of antibiotic.

Introduction

Despite the high caloric value and relative abundance of fatty acids and lipid materials in nature, there have been few investigations on the utilization of these compounds by soil actinomycetes. The metabolism of short-chain fatty acids and Kreb's cycle intermediates has been studied in *Streptomyces griseus* (1), and in *Streptomyces coelicolor* (5). Perlman (8) studied the utilization of lipid materials as a substitute for carbohydrate in the production of streptomycin by *S. griseus*. He found that animal and vegetable oils would not only replace glucose, but actually increased the yield of antibiotic. Later workers demonstrated that lipids and fatty acids substituted for glucose in the production of polyene antibiotics actually increased the yield up to 10-fold (4, 7).

Studies in this laboratory indicate that vegetable oils and constituents of these oils serve as a carbon and energy source for all *Streptomyces* isolates tested. This paper is concerned with the effect of naturally occurring oils and the fatty acid constituents of these oils on growth and antibiotic production by newly isolated organisms of the genus *Streptomyces*.

Materials and Methods

The organisms employed in these studies were fresh soil isolates belonging to the genus *Streptomyces*, as described by Waksman (10). In every case growth was for a period of 5 days at 27° C on a centrifugal shaker.

Dry weights were taken on cells collected by filtration and washed with fat-solvents to a constant dry weight. Antibiotic activity was determined by plate assay with ½-inch filter-paper disks. Assay plates were incubated for 18 hours and zones measured from disk edge to the outer edge of the zone of inhibition.

All fatty acids were neutralized with NaOH and were added without emulsification.

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Contribution from Kitchawan Research Laboratory, Brooklyn Botanic Garden, Ossining, New York.
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The basal medium used throughout for the growth of *Streptomyces* was composed of Bacto Soytone, 0.75%; Cerelose, 0.50%; and $CaCO_3$, 0.10%, at a pH of 7.2. The test organisms were an Actidione-sensitive strain of *Candida albicans*, and laboratory stocks of *Staphylococcus aureus* and *Bacillus subtilis*.

Results

The addition of corn oil to the basal medium utilized for the growth of various *Streptomyces* effected a considerable increase in both the growth and antibiotic production by these organisms. A routine study of 200 soil isolates indicated that, to some degree, all were stimulated in growth by the addition of 0.5% corn oil to the basal medium. For the majority of organisms the increase in amount of antibiotic produced was in proportion to the increase in total growth. However, with approximately 5% of the antibiotic-producing isolates, the *C. albicans* activity was disproportionately in excess of the growth stimulation. The results of a typical experiment are presented in Table I. As shown in this table, S6233 gave a 2-mm zone of inhibition on the basal medium and a 10-mm zone with the addition of corn oil. The increase in growth was from 3.40 to 4.36 mg/ml. This increase in amount of antibiotic appears to be excessive when compared with the increase in total growth.

TABLE I

The effect of corn oil on the growth and antibiotic production in a number of *Streptomyces* isolates

Isolate No.	Zone size in mm			Dry wt., mg/ml
	S. aureus	*B. subtilis*	*C. albicans*	
V761	3	4.5	0	2.84
V761 + oil	5	7	0	8.90
S1687	2	3	0	2.64
S1687 + oil	5	5	0	5.45
S2115	2	5	0	3.56
S2115 + oil	3	8	0	8.78
S6233	Sl.	2	2	3.40
S6233 + oil	1	3	10	4.36
K1926	1	2	0	2.56
K1926 + oil	2	4	0	4.40
S4286	0	0	7	2.12
S4286 + oil	0	0	12	3.36
S6881	0	0	0	2.76
S6881 + oil	0	0	0	5.56

A number of *Streptomyces* producing this high anti-*Candida* activity were grown with shaking in the basal medium in the presence and absence of 0.5% corn oil. Cellular material was separated by filtration through milk filter disks and the supernatant extracted with *n*-butanol. The butanol extract was chromatographed and bioautography suggested that, in all cases, the anti-

biotic produced on the addition of corn oil was the same as in the control. The antibiotics produced by these organisms fell into two distinct classes chromatographically. Further studies were initiated to determine the type of antibiotic produced. Comparative chromatography with authentic material suggested that Cycloheximide was one of the compounds (S6233). The other antiyeast compound was followed on chromatography by its U.V. absorption. The antibiotic was partially purified by chromatography and the characteristic absorption pattern of a polyene was obtained (2). This increase in amount of polyene antibiotic produced in the presence of fatty acids was noted earlier for Filipin (4) and for Fungichromin (7). No further purification of the antiyeast activities was undertaken.

Since corn oil stimulated growth and antibiotic production in the presence of carbohydrate, the level of corn oil necessary for maximum production of anti-*Candida* activity was established. The effect of adding increasing levels of corn oil to the basal medium can be seen in Table II. S6233 and S4286 are Cycloheximide producers and S4583 produces neither a polyene nor Cycloheximide. In both cases 0.5% corn oil added to the basal medium yielded a maximal antiyeast activity. The growth response to glucose and to corn oil singly and combined is demonstrated in Table III for a number of organisms chosen at random from soil isolates. The addition of glucose increased the total growth over that in the medium containing only Soytone. The addition of corn oil resulted in more total growth than glucose alone. The combination of equal amounts of the two compounds did not result in as much total growth as when corn oil was added singly. This would indicate that, for the organisms tested, glucose is inferior to corn oil as a carbon and energy source.

TABLE II

Effect of adding increasing levels of corn oil on antibiotic production

Isolate No.	Zone size (mm) with different percentages of corn oil added to basal medium						
	None	0.05	0.25	0.50	1.0	2.5	5.0
S6233*	5	8	12	15	14	14	14
S4286*	3	8	11	17	17	17	16
S4583	4	3	4	5	6	5	6

*Cycloheximide producers.

TABLE III

Comparison of glucose and corn oil as a carbon source for a number of *Streptomyces* isolates

Addition*	Level substrate, %	Dry wt. of cells (mg/ml medium) for isolate No.:								
		S2593	S54	V999	V1541	S283	S6800	S733	S1687	S220
Control	—	1.5	1.3	1.4	1.2	1.5	1.1	2.0	1.2	1.1
Glucose	1.0	1.9	1.8	2.5	2.7	4.1	1.7	3.2	2.1	1.9
Corn oil	1.0	3.4	3.6	5.8	3.8	6.5	4.3	4.5	1.9	2.7
Glucose + oil	0.5 0.5	3.1	2.7	4.6	4.2	5.3	3.9	5.6	2.6	2.6

*Basal medium was 0.5% Soytone.

In another experiment the concentration of corn oil was kept constant and glucose was added at levels of 0.1 and 0.5%. The three different responses are shown in Table IV. The results with V999 and S6800 are typical of the majority of organisms tested. The addition of low levels of glucose (0.1%) had little or no effect, while higher levels (0.5%) yielded an increase in total growth. Very few (less than 10%) of the organisms tested were stimulated to the extent that K153 is by the addition of glucose to the Soytone – corn oil medium.

TABLE IV

Influence of added glucose to organisms growing on a Soytone – corn oil medium

Isolate No.	Dry wt. of cells (mg/ml medium) with % added glucose of:		
	None	0.10	0.50
K153	2.90	3.24	4.16
V999	4.51	4.60	5.80
S6800	4.24	4.24	4.38

Other naturally occurring oils were tested to find whether this stimulatory effect is limited to corn oil. *Streptomyces* markedly stimulated in anti-*Candida* activity were chosen for study and the results are presented in Table V. The vegetable oils—olive, corn, and sesame—all yielded an equal increase in antibiotic production. The total growth in the presence of all the vegetable oils was virtually equivalent. The animal oil (sperm) was not significantly different from vegetable oil in effect on growth or antibiotic produced. The addition of mineral oil did not give a response significantly different from the control.

TABLE V

Effect of various oils on antibiotic production by representatives of the genus *Streptomyces*

Addition	Zone size (mm) for isolate No.:				
	S463	S3949	S467	S4286	S4322
None	2	0	1	8	0
Olive oil	8	7	8	16	12
Corn oil	10	6	8	16	12
Sperm oil	8	5	6	16	12
Sesame oil	10	8	6	16	12
Mineral oil	3	0	2	8	0

The constituents of corn oil were tested as replacements for the parent substance. Corn oil was added at a level of 2% for this experiment and the neutralized fatty acids were added at approximately the level corresponding to the amount of each in this level of corn oil. Results, in cell dry weight, are presented in Table VI. In general, the unsaturated fatty acids—oleic and linoleic (which predominate in corn oil)—were most effective. For a few, myristic acid yielded a greater growth response than corn oil.

TABLE VI

The effect of constituents of corn oil on the growth of various representatives of the genus *Streptomyces*

Addition	Level added in %	Dry wt. of cells (mg/ml medium) for isolate No.:											
		S6800	S6881	S5252	S7027	S6233	S220	S472	S733	S444	S3685	S5400	S3850
None	0	2.1	1.9	1.7	2.3	2.6	2.0	2.0	1.7	1.8	1.7	2.3	2.1
Corn oil	2.0	6.4	6.8	10.5	8.9	7.2	6.3	6.6	8.3	5.5	5.2	8.1	7.0
Myristic acid	0.4	2.7	2.9	2.6	2.7	3.8	2.8	2.0	4.5	3.5	11.7	11.6	8.5
Palmitic acid	0.2	2.6	2.6	2.8	2.6	3.5	2.7	3.3	3.3	2.1	2.2	4.0	3.2
Stearic acid	0.1	2.9	3.3	3.1	3.1	3.8	2.7	3.8	3.4	3.2	2.3	4.7	3.3
Oleic acid	1.0	4.2	4.0	7.4	7.7	6.1	5.1	6.4	3.8	5.4	4.9	5.2	5.6
Linoleic acid	1.0	5.9	5.3	7.4	6.8	7.4	6.7	5.9	5.8	7.2	6.4	7.0	4.1

Acetate and glycerol were also compared with corn oil and some related substances for growth stimulation. The results are presented in Table VII. Glycerol was an effective substrate for the growth of *Streptomyces*. This is in agreement with the findings of Pridham (9). As noted in an earlier report (5), acetate was not an effective substrate.

TABLE VII

Influence of various substrates on the growth of *Streptomyces* isolates

Addition	Dry wt. of cells (mg/ml medium) for isolate No.:				
	S220	S6881	V761	S1687	S2115
None	1.3	1.2	1.8	1.3	1.7
Corn oil*	2.2	2.8	3.0	1.3	4.7
Glucose	2.0	2.2	2.3	1.8	3.0
Glycerol	1.5	2.1	3.0	2.1	3.5
Acetate	1.6	1.5	2.2	1.7	2.4
Linoleic acid	1.3	1.9	4.0	1.5	6.5
Oleic acid	2.3	1.5	2.7	1.9	2.4

*Substrate concentration, 0.5%.

A number of known antibiotic producers were grown on the basal medium with and without the addition of corn oil. The antibiotic activity against three organisms was determined and is presented in Table VIII. The Cycloheximide-producing strain of *S. griseus* was the only one yielding a dispro-

TABLE VIII

Influence of corn oil on known antibiotic producers

Organism	Antibiotic	Oil	Zone size (mm) with different test organisms		
			S. aureus	*B. subtilis*	*C. albicans*
Streptomyces sp.	Litmocidin	−	10	10	0
		+	14	16	0
Streptomyces erythreus	Erythromycin	−	0	10	0
		+	1	10	0
Streptomyces venezuelae	Chloromycetin	−	4	7	0
		+	5	7	0
Streptomyces spirogriseus	Novobiocin	−	4	2	0
		+	8	5	0
Streptomyces halstedii	Carbomycin	−	3	6	0
		+	6	9	0
Streptomyces griseus	Grisein	−	1	5	0
		+	3	8	0
Streptomyces rimosus	Oxytetracycline	−	6	6	3
		+	9	9	7
Streptomyces chrysomallus	Actinomycin	−	6	3	0
		+	10	9	0
Streptomyces griseus	Streptomycin + Cycloheximide	−	2	9	1
		+	2	10	17

portionate increase on the addition of corn oil. Although no known polyene producers were available, it is expected from the results of Brock (4) and McCarthy (7) that a corresponding increase would result.

Discussion

The actinomycetes play a prominent role in the decomposition of protein materials in the soil. Earlier workers (3) have found that the actinomycetes are not particularly adept at oxidizing all sugars, although the common sugars, e.g. glucose, are oxidized by most members of the genus *Streptomyces*. All of 200 randomly chosen *Streptomyces* were stimulated in growth by the addition of 0.5% corn oil to the basal medium. In organisms producing polyene or Cycloheximide antibiotics a considerable increase in antibiotic activity resulted on the addition of corn oil or unsaturated long-chain fatty acid.

The level of vegetable oil necessary for maximal stimulation is in the substrate (0.5%) range although low levels of the individual fatty acids do stimulate growth in some organisms. Experiments with corn oil as the sole source of carbon and energy indicate that, for most organisms tested, fatty acids not only replace sugars but are superior to them.

Since the major constituents of vegetable oils are similar both qualitatively and quantitatively, they had an equivalent effect on growth and antibiotic production. The animal oil (sperm) is not significantly different in constitution and, therefore, was much the same in effect on the growth of the *Streptomyces*.

Oleic acid, linoleic acid, and glycerol were all effective replacements for the vegetable oil. These results are similar to results of Khan and Chughtai (6) working with *Aspergillus niger* and *Penicillium notatum*. These fungi, utilizing cottonseed oil as a sole carbon source, selectively attack the unsaturated oleic and linoleic acid and do not oxidize the saturated fatty acid constituents.

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MICROBES AND NITRATES IN SOILS FROM VIRGIN AND YOUNG-GROWTH FORESTS[1]

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Abstract

Penicillium spp. predominated in samples of forest soils except occasionally at depths of more than three inches, when *Mucor* and *Aspergillus* spp. sometimes were more abundant. Incubation for 30 days at 28° C and 50 per cent water-holding capacity frequently increased the percentage of *Mucor* spp. as well as *Penicillium* spp. *Mucor* spp. were consistently more prominent in soils associated with alder than for other coastal soils. *Mucor* and *Aspergillus* spp. also appeared often in soil from stands of ponderosa pine growing east of the Cascades.

The greatest concentration of nitrogen as NO_3^- in unincubated soils was found in a young red alder stand. Samples of soil from stands of virgin coastal redwood showed no nitrate nitrogen. Soils from stands of virgin Sitka spruce, however, showed considerable nitrate content, which increased markedly with incubation.

With few exceptions, bacteria and actinomycetes were most numerous in F layers of soil. Incubation greatly increased these populations in most soils.

Introduction

Knowledge of the microflora in soil and its role in the formation and decomposition of humus is becoming increasingly important in forest management. For the Pacific northwest, there is only meager information on microflora of forest soils and of changes that take place following logging of forests and burning of slash. This paper presents data on microflora of soils from virgin and from young-growth forests.

Powers (1) presented evidence that fermenting and humified layers of nine soil profiles from the northwest forests are of major importance in supplying nutrients for forest growth. Powers and Bollen (2) later stated that nutrient-supplying power, especially for bases and nitrates, is centered in F and H layers of forest soils. They also found microorganisms to be most numerous in these layers.

Vandecaveye and Baker (4) found about five times as many microbes in Palouse silt loam, an agricultural soil, as in Melbourne silt loam, a forest soil. Microflora of Palouse soil consisted of a large number of bacteria and actinomycetes and a small number of fungi. Microflora of Melbourne soil under a pine forest was composed of about seven times as many molds and only about one-sixth as many bacteria and actinomycetes as in Palouse soil. There was 4.4 per cent organic matter in the A horizon of Palouse soil, while the A horizon of Melbourne soil contained 8.1 per cent organic matter.

Tresner *et al.* (3) found that soils of pioneer forests in southern Wisconsin generally yielded few species of molds, while soils from climax forests contained many species. However, when comparison was based on index of forest con-

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tinuum, the greatest number of species did not occur in the climax stand, but in an intermediate range. They also found that the soil environment of the forest continuum not only had determined the number and kinds of fungi present, but its influence was reflected in the distribution of entire groups of microorganisms, such as *Penicillium* and some Mucoraceae, including *Rhizopus*. This paper, and others, indicate that comparative averages in studies of soil microbiology can be valuable, but such comparisons should be made with discretion.

Methods and Procedures

Samples of profiles of the forest soils listed in Tables I, II, and III were composites of three subsamples from each horizon. Each subsample consisted of material from an area one foot square, three being representatively selected within areas of about 20 square feet. Each horizon was sampled with precaution to avoid contamination from other horizons. Samples were brought to the laboratory in sterile bottles or cans as soon as possible. In no instance did more than three days elapse before passing the soil through a sterilized 10-mesh screen, starting analysis, and pouring dilution plates.

Determinations of pH were made with a glass electrode, using suspensions of 20 parts of sterile distilled water to one part of organic layers, and five parts of water to one part mineral soil, water-free basis, immediately after these had been prepared for making the serial dilutions for plate counts. Triplicate plates of appropriate dilutions were poured with peptone–glucose agar acidified to pH 4 for fungi, and with sodium albuminate agar for bacteria and actinomycetes. Although the use of acidified agar is known to restrict the growth of certain molds, this may be less important in studies with acid soils. Counts were made on original samples and also on samples incubated 30 days at 28° C with moisture content adjusted to 50 per cent of water-holding capacity. Moisture capacity was calculated from weight of water retained by samples in large Gooch crucibles wetted from below and drained to constant weight in a saturated atmosphere. Fifty per cent of this value corresponds to about field capacity as calculated from $\frac{1}{3}$ to $\frac{1}{2}$ atmospheric tension. Soil molds of only the three most commonly occurring genera generally were identified. Nitrate (NO_3^-) nitrogen was determined on clarified filtrates of 1:5 soil suspensions by the phenoldisulphonic acid method.

Results

Soil characteristics, locations, and microbial analyses are presented in the first three tables.

Molds Present

All forest soils from the coastal side of the Cascade Range were moderately acid, except for soil from a young-growth Douglas fir forest, about 30 years old, near Lents, Oregon, which approached neutrality. In general, L and F horizons were slightly more acid than were horizons A and B. H layers were not distinguishable.

Rhizopus spp. and other mucors, and *Aspergillus* spp., made up only a small percentage of the microflora of soils from stands of virgin Douglas fir, while

TABLE I

Molds, bacteria, and actinomycetes in forest soil profiles, stands west of Cascade Mountains

Horizon	Depth, in.	% soil moisture	% water-holding capacity	Soil pH		Molds								Bacteria-actinomycetes			
						Total		Mucors		*Aspergillus*		*Penicillium*		All		Actinomycetes	
				Original	Incubated 30 days	Original	Incubated	Original	Incubated	Original	Incubated	Original	Incubated	Original	Incubated	Original	Incubated
						Thousands*		Per cent†		Per cent†		Per cent†		Millions*		Per cent†	
Virgin Douglas fir on Aiken silty clay loam at Corvallis, Oregon																	
F_1	0–1.0	53	364	6.1	6.5	25	1080	0	12	0	3	40	7	4.4	188	8	14
F_2	1–1½	45	523	4.3	4.7	148	3450	9	0	0	0	88	12	2.1	19	14	29
A_1	1½–4	33	95	6.5	6.1	23	52	0	0	0	0	87	85	1.5	7	35	40
A_2	4–16	30	93	6.3	6.1	20	33	0	1	0	0	80	88	0.6	5	49	83
B_1	16–31	23	90	5.9	5.6	4	21	0	0	0	0	43	88	0.1	1	55	40
B_2	31–48	36	107	6.1	5.5	0.2	0.1	0	0	0	0	50	92	0.003	0.4	29	0
Virgin Douglas fir on Olympic silty clay loam at Corvallis, Oregon																	
F_1	0–1	14	125	5.6	5.7	10	200	0	1	0	35	10	0	0.5	13	60	16
F_2	1–2	20	460	5.9	7.0	17	450	0	9	0	0	35	0	1.8	158	39	17
A_1	2–5	23	84	6.9	6.9	43	133	0	1	0	0	98	88	2.8	10	45	81
A_2	5–20	22	80	6.5	6.2	9	20	0	0	0	0	95	90	3.5	5	46	67
B	20–40	32	100	6.0	5.6	0.2	0.3	0	20	0	0	33	41	0.05	0.1	63	36
Virgin Douglas fir on Parkdale loam at Parkdale, Oregon																	
F_1	0–3	33	265	6.6	6.9	140	97	4	20	0	0	2	80	42	160	47	97
F_2	3–5	27	87	6.6	6.6	43	52	2	0	0	4	84	13	12	16	28	93
A	5–11	25	73	6.8	6.9	1	6	0	45	1	1	37	19	1.6	12	31	85
B	11–17	25	70	6.8	6.8	3	11	1	90	1	1	5	8	2.7	11	44	86
Young-growth Douglas fir on Salem gravelly fine sandy loam at Lents, Oregon																	
F_1	0–1	18	108	6.4	6.2	161	—	11	—	1	—	81	—	3.6	34	29	93
F_2	1–3½	21	93	6.3	6.2	45	90	4	29	1	3	89	68	12	14	32	84
A	3½–6½	17	71	6.3	6.7	22	57	5	19	4	0	88	79	6	9	45	81
B	6½–12	16	61	6.4	6.7	0.9	5	7	77	4	1	6	17	0.7	3	44	94
C	12–23	16	52	6.4	6.7	0.5	0.7	20	46	18	6	25	33	0.2	0.9	40	90

*Per gram of soil, dry basis.
†Based on total observed for the group.

TABLE II

Molds, bacteria, and actinomycetes in forest soil profiles, stands along Pacific coast

Horizon	Depth, in.	% soil moisture	% water-holding capacity	Soil pH		Molds								Bacteria-actinomycetes			
						Total		Mucors		*Aspergillus*		*Penicillium*		All		Actinomycetes	
				Original	Incubated 30 days	Original	Incubated	Original	Incubated	Original	Incubated	Original	Incubated	Original	Incubated	Original	Incubated
						Thousands*		Per cent†		Per cent†		Per cent†		Millions*		Per cent†	
Virgin Sitka spruce on sandy loam at Otter Rock, Oregon—summer collection																	
L	0–½	43	370	5.9	6.1	182	—	2	0	1	0	10	—	0.4	11	62	65
F1	½–3	48	268	4.9	4.7	322	1.2	4	2	0	0	93	91	2	0.6	52	71
F2	3–23	25	91	5.3	5.3	200	0.1	12	15	0	0	80	40	0.2	0.4	71	30
A	23–26	20	69	5.9	5.4	29	0.05	0	2	14	0	82	29	0.04	0.7	10	11
B	26–38	3	37	6.4	5.0	0.1	0.003	0	7	55	0	45	75	0.001	0.2	0	21
Virgin Sitka spruce on sandy loam at Otter Rock, Oregon—winter collection																	
L	0–½	198	435	5.7	5.1	98	171	6	0	0	7	78	18	31	19	3	11
F1	½–3	166	315	5.1	4.9	159	175	1	1	0	10	54	19	32	0.8	8	57
F2	3–9	52	120	5.0	5.1	66	102	3	6	0	0	17	26	52	0.4	1	90
F3	9–23	46	97	5.7	5.9	3	9	16	4	3	11	16	58	5	1.0	1	54
A–B	23–28	25	61	5.5	6.2	17	37	6	1	0	0	2	36	2	2.6	2	16
C	28–35	6	37	5.8	5.8	0.2	0.2	0	0	0	95	0	75	0.002	0.01	5	20
Virgin Coast redwood on sandy loam at Crescent City, California																	
L	0–½	12	330	5.2	5.7	25	11	0	2	0	0	84	92	0.1	2.3	24	84
F1	½–1.0	26	102	6.2	5.9	183	0.2	3	3	0	0	30	28	21	5.2	70	68
F2	1–7	21	78	6.3	5.8	141	0.2	2	10	0	0	4	28	7	1.9	80	88
A	7–13	22	74	6.1	5.5	22	0.04	25	50	1	0	70	50	1.2	0.8	70	66
B	13–17	14	65	6.1	5.3	11	0.1	35	60	0	0	55	7	0.5	44	50	5
Shore pine on Blacklock sandy loam at Mercer Lake, Oregon																	
L	0–½	177	392	4.5	4.3	270	1050	0	0	0	0	52	67	33	2	1	24
F	½–1.0	159	374	4.9	5.0	700	1750	7	11	0	0	40	92	6	2	14	10
H	1–2	10	100	4.5	4.4	1380	225	0	2	0	4	47	39	1	0.37	1	5
A1	2–3	14	51	4.6	4.9	225	175	35	3	1	20	9	43	0.43	0.33	5	8
A3	3–10	8	38	5.1	5.8	56	53	0	0	2	80	18	12	0.32	0.38	3	5
B1	10–16	5	38	5.4	6.3	4	87	0	0	18	57	16	24	0.05	0.14	5	12
B2	16–28	5	36	5.5	6.4	4	85	14	0	4	0	27	39	0.24	0.25	0.1	3
C	28–48	3	31	5.5	6.9	1	3	0	0	0	0	13	23	0.28	0.58	0.1	0.4
Young red alder on recently exposed Cretaceous sandstone at Otter Rock, Oregon																	
F	0–1	79	305	4.9	5.7	13	10	32	29	0	0	64	68	0.4	0.3	21	38
A	1–7	51	107	4.3	4.3	5	6	22	27	0	5	66	64	0.1	3	6	5
B1	7–14	45	62	5.0	4.9	9	5	12	25	6	0	70	40	0.1	1	19	18
B2	14–32	34	67	4.9	4.7	3	4	17	7	0	13	50	60	0.1	1.3	16	15
C	32–48	47	61	5.0	4.7	11	6	2	0	0	0	66	14	0.2	2.4	8	4

*Per gram of soil, dry basis.
†Based on total observed for the group.

TABLE III

Molds, bacteria, and actinomycetes in forest soil profiles, stands east of Cascade Mountains

Horizon	Depth, in.	% soil moisture	% water-holding capacity	Soil pH		Molds								Bacteria-actinomycetes			
						Total		Mucors		*Aspergillus*		*Penicillium*		All		Actinomycetes	
				Original	Incubated 30 days	Original	Incubated	Original	Incubated	Original	Incubated	Original	Incubated	Original	Incubated	Original	Incubated
						Thousands*		Per cent†		Per cent†		Per cent†		Millions*		Per cent†	
Virgin ponderosa pine on sandy loam at Camp Sherman, Oregon																	
F	0–½	2	254	4.5	4.5	2	575	—	—	—	—	—	—	0.04	0.05	2	0
A	½–7	4	48	6.6	6.5	157	375	—	—	—	—	—	—	0.08	1.1	16	36
B	7–15	4	47	7.2	6.9	13	21	—	—	—	—	—	—	1.0	2.7	15	25
Young-growth ponderosa pine on Helmer silt loam at Helmer, Idaho																	
F_1	0–½	68	248	6.3	6.7	165	—	24	—	1	—	63	—	37	22	31	82
F_2	½–2	41	97	6.4	6.5	131	84	0	24	1	1	47	38	33	10	50	76
A	2–8	21	46	5.9	6.3	15	16	1	1	10	2	65	84	1.8	30	27	80
B	8–17	18	36	6.2	6.5	0.7	6	13	3	13	0	18	95	1.2	3	35	84
C	17–25	17	34	6.2	7.2	0.3	9	4	16	4	0	33	35	0.4	3	32	75
Western juniper on fine sand at Redmond, Oregon																	
L	0–½	2	212	5.4	6.1	7	10	—	—	—	—	—	—	0.1	22	4	9
F	½–2	8	40	7.4	7.5	9	13	—	—	—	—	—	—	3.4	7	15	63
A	2–14	9	51	7.6	7.5	4	6	—	—	—	—	—	—	0.9	18	32	11
B	14–18	6	47	8.0	7.6	2	4	—	—	—	—	—	—	0.5	15	20	4

*Per gram of soil, dry basis.
†Based on total observed for the group.

species of *Penicillium* strongly predominated in all samples. Soils from stands of young-growth Douglas fir showed a noticeable increase in the percentage of mucors and a lesser increase in *Aspergillus*. *Penicillium* predominated.

Incubation for 30 days at 28° C and at 50 per cent water-holding capacity tended to decrease the incidence of *Penicillium* in F horizons and increase it in lower horizons. There were exceptions, particularly in soils from forests of young-growth Douglas fir.

In most instances, incubation increased the percentage of actinomycetes. Actinomycetes also were more prominent in soil collected during the summer months under virgin Sitka spruce than in winter-collected samples from the same stand.

Mucor spp. were consistently more prominent in soils under red alder than in most other coastal soils. This was particularly true of F and A horizons. In soils associated with coastal redwood, *Mucor* spp. were most prominent in A and B horizons.

For soils east of the Cascades, the horizons from the young-growth (18 years old) stand of ponderosa pine showed comparatively high abundance of *Mucor* and *Aspergillus* spp.; however, *Penicillium* spp. still predominated, as in samples of west-side soil. Soils from east of the Cascades generally were more acid in L and F horizons than were west-side soils, but deeper horizons were more alkaline. Surprisingly enough, there was not a corresponding increase in actinomycetes.

The preceding statements necessarily are generalized, because there was considerable difference in types of soil. Climatic influences, however, were similar, as with soils from virgin stands of Douglas fir or stands of Sitka spruce and red alder. Climatewise, Parkdale and Lents soils also provide a good basis for comparison, as do all east-side soils.

Microbial Populations

Relative numbers of molds, bacteria, and actinomycetes in samples of soil, as determined by counts in dilution plates, are shown in Tables I, II, and III, and reveal two significant trends. First, incubation for 30 days at 28° C and 50 per cent water-holding capacity generally increased mold populations. Secondly, counts of both mold and bacteria–actinomycetes for the red alder stand were extremely low; the soil on which this stand was growing, however, developed during only 20 years on cretaceous sandstone exposed in an abandoned railroad cut. Counts also were low for soils associated with virgin ponderosa pine and western juniper. The numbers of bacteria and actinomycetes generally increased in the incubated soils. Exceptions include the marked decrease in counts of bacteria in the incubated winter-collected L and F horizons of the Sitka spruce soil, although the number of actinomycetes increased extensively. Similar changes, but less extensive, occurred with the Helmer soil. Incubation decreased the number of bacteria in the upper horizon samples from the shore pine soil also.

Summer-collected soils from stands of virgin Sitka spruce showed a much greater population of molds than did winter collections. Incubation caused a decided decline in molds in summer-collected soils, but increased the population of the winter samples. These results could be expected from the influence of incubation.

Soils from forests of coast redwood and red alder gave low counts of molds and bacteria–actinomycetes. Incubation did not increase the number significantly; there was a decline with soil from redwood forests except for the L horizon.

With F horizons, the greatest mold count, 700,000, occurred under coastal shore pine; the next greatest, 322,000, was for soil collected in summer under stands of virgin Sitka spruce. The smallest mold count of 2000 was found in soils from stands of virgin ponderosa pine east of the Cascades. There was a general decrease in number of molds with depth of sample. All counts consistently were greatest in F horizons.

Highest total bacterial counts, including actinomycetes, were found in the F_1 layer from virgin Douglas fir forest near Parkdale, and from soil of the F_1 layer collected in winter under virgin Sitka spruce. Lowest count was shown by the L horizon of soil from virgin stands of coast redwood.

TABLE IV

Nitrate nitrogen in forest soil profiles

	Nitrate N, p.p.m.		
	Original sample	Incubated sample	
		30 days	240 days
Virgin Douglas fir, Parkdale loam			
F_1	38	12	
F_2	22	6	
A	0	2	
B	0	3	
Young-growth Douglas fir, Salem gravelly fine sandy loam			
F_1	80	135	
F_2	17	31	
A	26	4	
B	32	0	
C	10	0	
Virgin Sitka spruce, winter sandy loam			
L	0	517	666
F_1	0	125	182
F_2	0	0	0
F_3	0	7	12
A–B	0	0	0
C	0	0	0
Young red alder, Cretaceous sandstone			
F	113	0	
A	38	6	
B_1	10	22	
B_2	0	5	
C	0	0	
Young-growth ponderosa pine, Helmer silt loam			
F_1	38	0	
F_2	26	0	
A	35	2	
B	0	1	
C	0	0	

Nitrate Nitrogen

Nitrates were found in only five of the soils, as shown in Table IV. No nitrites were detected by the alpha-naphthylamine and sulphanilic acid method.

The greatest concentration of nitrogen as NO_3^- in the original samples, 113 p.p.m., was obtained in the F horizon under red alder. The concentration decreased to zero after incubation for 30 days. The unusually high value of nitrate found in the original sample may be attributed in part to the nitrogen-fixing ability of *Streptomyces alni* or other microbe associated with alder roots. Root nodules of this symbiont were abundant on the stand sampled. The nitrogen fixed sooner or later becomes ammonified during decomposition of fallen leaves and other residues, and any ammonia not absorbed by roots is subject to nitrification. The reason for the complete disappearance of nitrate after incubation is not clear. It could result from assimilation by microbes decomposing relatively fresh organic matter of wide carbon-to-nitrogen ratio.

Soils from stands of young-growth ponderosa pine showed before incubation a maximum of 38 p.p.m. in the F_1 layer, and Parkdale loam under virgin Douglas fir was equally high. The gravelly fine sandy loam from the stand of young-growth Douglas fir showed a maximum of 80 p.p.m. in the F_1 horizon.

Incubation for 30 days of the winter-collected samples from virgin Sitka spruce increased NO_3^- nitrogen content from 0 to 517 p.p.m. in the L layer. Nitrogen content further increased to 666 p.p.m. after eight months. This extraordinarily high accumulation of nitrate is unusual for its occurrence at pH 5.1. While the decomposition of litter undoubtedly supplied essential calcium and other bases, the nitrifying organisms nevertheless must have been adapted to an environment more acid than pH 5.6, which generally is considered near the lower limit for nitrification in agricultural soils.

Soils from virgin coast redwood showed no nitrate nitrogen in the original samples and virtually none after 30 days' incubation. Summer-collected soils from virgin Sitka spruce and soils under shore pine also gave negative results in tests for nitrate nitrogen, both before and after 30 days' incubation. The temperature and moisture during the summer likely had already induced extensive decomposition and nitrification when samples were taken, and any ammonium or nitrate formed had been assimilated by roots and microbes. No tests for nitrate nitrogen were made for soils associated with virgin Douglas fir collected near Corvallis, Oregon.

The significance of nitrification in soils of coniferous forests requires elucidation. The decomposition of nitrogenous organic matter liberates ammonium, and possibly nitrogen in this form may have greater desirability for conifers, as is true for certain crop plants.

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DEATH OF ESCHERICHIA COLI FROZEN ON CELLOPHANE AND ON MEMBRANE FILTERS[1]

HAROLD W. BRETZ

Abstract

Because *Escherichia coli* stored at −9° C on cellophane or membrane filter disks die similarly to cells in frozen suspensions, it seems unlikely that mechanical crushing or concentrated solute is responsible for the major part of freezing death. Increased survival of high concentrations of cells regardless of the method of storing implies an interaction between cells prior to freezing. The age of the cells plays a part. The results may be interpreted in terms of an exchange or loss of stimulatory or inhibitory substances in small amounts from individual cells either before or after frozen storage.

Introduction

The cause of death of bacteria from freezing is still obscure. Plant cells are readily ruptured by growing ice crystals; red blood cells are susceptible to the ensuing high concentration of salts; rickettsiae die under certain conditions of freezing unless replenished with diphosphopyridine nucleotide after thawing. Other factors than mechanical ones seem to account for bacterial death at −22° C since "there is no correlation between the physical strength of bacterial cells and their susceptibility to the lethal effects of freezing and thawing" (9). Intracellular ice has not been observed in bacteria, but has been interpreted as a mechanism of death (13, 14). Harrison (7) showed that successive exposures to −22° C in 4.6 *M* NaCl, which does not solidify at that temperature, gave a series of pendant curves similar to those found in broth suspensions which do freeze. He attributed death to concentrated solutes. In the present work *Escherichia coli* cells were placed on cellophane strips and on membrane filters at concentrations expected to provide cells separated from one another during subsequent frozen storage. The results argue against both mechanical disruption and concentrated solute as major causes of bacterial freezing death.

Materials and Methods

Inocula from a refrigerated stock slant of typical *Escherichia coli* (Illinois Institute of Technology strain 61–615) were smeared on plates of HYT agar (2% heart infusion agar, 0.5% yeast extract, 0.5% Trypticase, 0.75% additional agar, 0.1% dextrose). After incubation overnight at 37° C, four or five typical colonies were pooled in 5 ml of pH 7.0, 0.067 *M* Sorensen's buffer (3.53 g KH_2PO_4, 5.79 g anhydrous Na_2HPO_4, 1000 ml distilled water) and loopsful spread with a glass rod on HYT plates to provide sufficient test cells after the desired length of incubation. The resultant confluent growth was scraped off with a platinum loop to centrifuge tubes of buffer. After they had been washed three times in buffer with centrifugation, the cells were suspended

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in large quantities of buffer to give the desired optical density at 610 mμ with a Coleman Model 14 spectrophotometer. Suspensions were tubed in 1-ml amounts in 18×150 mm tubes covered with flat-topped aluminum caps and placed at −9° C. Thawing was accomplished by adding 9 ml of buffer at room temperature.

Washed cellophane strips (L×18 Zephyr Casings, Visking Co., Chicago, Ill.), 12×80 mm, were autoclaved under distilled water in petri dishes and transferred singly to dried 3% plain agar – water plates and left for several hours at 37° C to absorb the excess water. Then 0.01 ml of appropriate cell dilutions was added to cover most of the strip without spilling over onto the agar. After several minutes, the excess liquid having been absorbed, the strips were transferred singly to 18×150 mm test tubes or placed three in a petri dish. They were then stored at −9° C. Thawing was accomplished by adding 10 ml buffer to the still cold tubes, or the plates were warmed and the strips were transferred to test tubes of 10 ml buffer. The two procedures gave the same results. Samples (1 ml) were then diluted in buffer using the general procedures of *Standard Methods for the Examination of Dairy Products* (1) and plated in triplicate in HYT. After 24 to 36 hours at 37° C the colonies were counted using a Quebec counter.

For storage on membrane filter disks (type HA, Millipore Filter Corp., Bedford, Mass.), appropriate dilutions of washed cells were impinged on the filter with suction and rinsed with 10 to 15 ml buffer. The semidry disks were removed to sterile plastic dishes and stored at −9° C. Duplicate disks were warmed at room temperature and transferred to sterile filter pads in petri dishes containing 2 ml of HYT broth. After incubation at 37° C for 18 to 20 hours, the disks were placed on filter pads of dilute methylene blue and the resultant stained colonies counted through a 10.5× binocular magnifier.

Nuclear stains were made with a loopful of bacterial suspension and a loopful of modified Chance (4) stain (1 ml 2% acid fuchsin, 0.3 ml 2% uranyl nitrate, 0.1 ml 1% cerrous sulphate) stirred on the slide until dried.

Results

Survival patterns of cells 24 hours old at concentrations of 2.2×10^9, 2.4×10^7, and 2.8×10^5/ml on cellophane strips at −9° C are shown in Fig. 1. All concentrations show "typical" curves similar to those found for frozen buffer suspensions: a rapid initial rate of death followed by a slower rate of death during storage (12). Since there was no appreciable difference whether the tubes were thawed before or after the addition of diluent, the results are the averages of six replicates.

A second experiment was similar to the first except that inoculum 48 hours old was used and the cells were stored in frozen buffer as well as on cellophane (Table I). In the most concentrated suspension, survival was greater than on cellophane. The average distance between evenly distributed cells on cellophane would be about 4 μ, but the method of applying the inoculum probably makes this distance much smaller; the cells may clump in drying. At the lower concentrations it is common to find a great deal of variability between replicates. Whereas the coefficient of variation for non-frozen replicates is less than 10%, it may be from 10 to 15% for frozen cells (3).

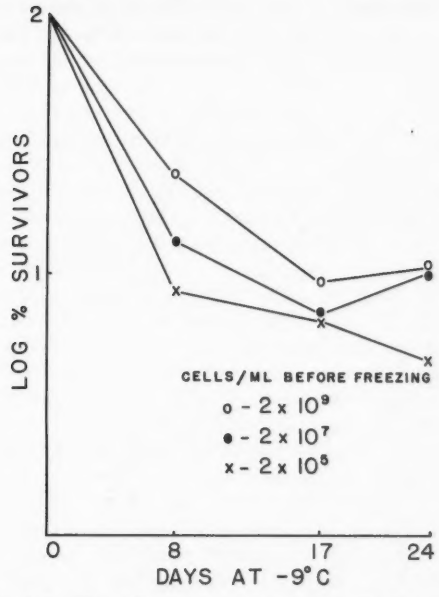

FIG. 1. Survival of *E. coli* at three concentrations on cellophane strips after storage at −9° C. Each point represents the average of six replicates with each replicate being the average of three plates.

TABLE I

Survival of *E. coli* at −9° C on cellophane strips and in buffer suspensions

	% survival*					
	2.3×10⁹†		2.3×10⁷†		2.5×10⁵†	
Storage time, days	Cello-phane	Suspen-sion	Cello-phane	Suspen-sion	Cello-phane	Suspen-sion
7	53	60	60	26	41	3.9
15	38	48	20	3.5	26	0.72
22	29	31	6.2	1.7	7.8	0.13

*Each figure represents the average of six replicates with each replicate being the average of three plates.
†Initial number of cells/ml suspension.

In order to assure separation of cells, several low concentrations of cells 4, 12, 24, or 48 hours old were impinged onto membrane disks with the results shown in Fig. 2. Again we see that the more cells in the original inoculum, the greater the survival, and that the age of the cells seems to influence survival. The nuclear configurations of these non-frozen cells differed with the ages: cells 12, 24, and 48 hours old were 60 to 80% uninucleate, while those 4 hours old were only 47%, but there was no correlation with the sub-

sequent survival behavior. Others (17, 19) have shown that the survival curves at −22° C or susceptibility to chilling at 4° C are not due to a fraction being selected from a heterogeneous population.

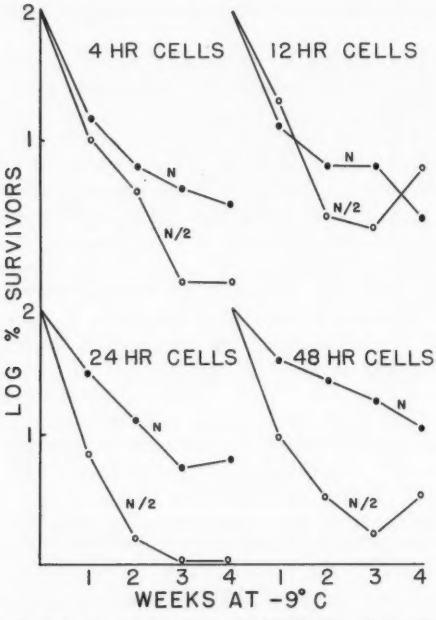

FIG. 2. Effect of age and concentration on survival of *E. coli* on membrane filter disks after storage at −9° C. *N*, the original number per disk (less than 400), for each respective age of cells; *N*/2, half the original number. Each point represents the average of duplicate disks.

Discussion

Bacterial cells frozen on cellophane or membrane filters give survival patterns similar to cells in frozen suspensions: a rapid initial rate of death followed by a slower rate. The extent of death is dependent upon the physiological state of the inoculum. The fact that concentrated cells almost always survive better than more dilute ones implies that there is an interaction between cells before or after storage which influences survival. On membranes, neither mechanical crushing nor concentrated solute can be responsible for death, but the effect of dehydration may be operative. The 2×10^9 cells/ml survived slightly better in frozen suspension than on cellophane, whereas the 2×10^7 and 2×10^5 cells/ml were just the opposite, but the differences in experimental methods make it difficult to know which concentrations to compare. On membranes there should be no direct storage interaction between cells; yet the general shape of the storage curves is similar to that for frozen suspensions, where the cells are probably in intimate contact.

Mazur (13, 14) interprets subzero death in terms of physical factors, especi-

ally intracellular ice crystals, since suspending menstrua which do not freeze do not cause death. Intracellular ice is not precluded as a cause of death of cells on membranes. However, these results can also be interpreted in terms of an exchange or loss of cellular components during preparation of the suspensions or changes during storage within individual cells (3). The washed cells are not in a growth medium, but a slow rate of endogenous metabolism could lead to a kind of unbalanced growth or accumulation of toxic products. A similar interpretation has been given for *Pasteurella pestis* stored at 5° C (16). Harrison (8) and Strange *et al.* (21) have emphasized the role of cannibalism and endogenous metabolism in the survival of *Aerobacter aerogenes* at growth temperatures.

Since it is known that *E. coli* loses ribonucleic acids in 0.067 *M* phosphate buffer (5) and *Bacillus megaterium* leaches ribose polynucleotides in 0.06 *M* phosphate (6), any substance which could prevent such loss might conceivably increase survival. Glycerin (10) and certain sugars (15) may act non-specifically in this way. A lecithin-like viability protective factor for freeze-drying has been isolated from *Brucella* (2), and freeze-dried *E. coli* can be protected by cell exudates (18). Kohn (11) has shown that *E. coli* frozen in growth media become sensitive to lysozyme lysis. Non-lethal injury to frozen bacteria which can be overcome by Trypticase in the plating medium has been demonstrated by Straka and Stokes (20). Thus specific substances in small amount may act as stimulators or inhibitors to freezing death and these substances may arise from the cells themselves, dependent upon their physiological state, their growth medium, and the storage menstruum. Preliminary trials indicate that such substances are present in frozen *E. coli.*

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CARBOHYDRATE METABOLISM IN MICROCOCCUS RADIODURANS[1,3]

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Abstract

The essential characteristics of the primary and secondary pathways of carbohydrate catabolism in a radiation-resistant micrococcus were studied by means of the radiorespirometric tracer method. Measurements of relative rates and total percentage of $C^{14}O_2$ produced from different carbon atoms of specifically labelled acetate, pyruvate, glucose, and gluconate, together with determination of the C^{14} incorporation into cells, indicated that glucose was utilized mainly by the Embden–Meyerhof–Parnas glycolytic pathway in conjunction with the tricarboxylic acid cycle.

Approximately 14 per cent of the glucose appeared to be catabolized via phosphogluconate decarboxylation.

Introduction

In a previous report (7) the utilization of simple carbohydrates, intermediates of the tricarboxylic acid (TCA) cycle, and amino acids by *Micrococcus radiodurans*[3] was studied by means of manometric and radioactive tracer techniques. In view of the unique resistance displayed towards high doses of radiation (1, 3), it appeared desirable to examine in detail the nature of the pathways of carbohydrate catabolism functioning in this organism.

In the present work, the nature of the secondary catabolic pathways of carbohydrate metabolism in *M. radiodurans* has been studied by means of a series of radiorespirometric experiments employing test substrates such as C^{14} specifically labelled acetate and pyruvate, which are generally considered as the key intermediates of glucose catabolism. Experiments were carried out in a chemically-defined growth medium in order to minimize variations in replicate experiments.

Materials and Methods

Cell Suspensions

Micrococcus radiodurans was grown under aeration for 24 hours at 30–34° C in a chemically defined medium (7). The cells were collected from the medium by centrifugation at 3000 r.p.m. For respirometric studies, the washed cells were resuspended in a synthetic medium basically the same as the chemically defined medium above from which the specific components to be used as substrates were omitted, and the suspension adjusted by means of optical density

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Contribution from the Departments of Bacteriology and Chemistry, Oregon State College, and Oregon Agricultural Experiment Station (Technical Paper No. 1355), Corvallis, Oregon.

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[3]Tentative nomenclature proposed for this radiation-resistant microorganism.

to contain approximately 3 mg of cells (dry weight) per ml. This was equivalent to about 10 times the cell concentration of the original 24-hour culture.

C^{14}-Labelled Substrates

Glucose-1-, -2-, and -6-C^{14}; pyruvate -1-, -2-, and -3-C^{14}; acetate-1- and -2-C^{14}; and sodium gluconate-1-C^{14} were obtained from commercial sources. Glucose-3,(4)-C^{14} was prepared in this laboratory by the method of Wood (9). D-Gluconate-2-, -3,(4)-, and -6-C^{14} were prepared according to the method of Moore and Link (6). All labelled substrates were examined for their respective purities by means of paper chromatography and were adjusted to desirable specific activity prior to administration.

Radiorespirometric Experiments

Radiorespirometric studies of C^{14} specifically labelled glucose, gluconate, pyruvate, and acetate were carried out according to the method of Wang (8). A 10- or 15-ml quantity of cell suspension was added to each flask after the desired test substrate had been placed in the sidearm (Tables I, II, and III). In experiments employing gluconate, 3.75 mg unlabelled glucose (approx. 21 μM.) was added to each flask in addition to the C^{14} specifically labelled gluconate. The experiments were generally allowed to continue for 7–10 hours. (Tests revealed no contamination of the culture at the end of the 10-hour period.) Following each experiment, cells and media were separated by centrifugation. Cells were dried over P_2O_5 in a vacuum desiccator at room temperature and weighed.

Radioactivity Measurements

The cells, medium, and substrate solutions were assayed for radioactivity as described previously (7, 8).

Results and Discussion

The radiorespirometric study of carbohydrates utilized in a biological system indicates the relative rates of combustion of substrate carbon atoms to CO_2, which reflects directly the nature of catabolic pathways functioning in the system. The catabolic mechanism in a microorganism may be understood as consisting of a primary pathway which is responsible for the breakdown into small fragments and a secondary pathway which serves for the combustion of the degradation products. When an organism is incubated in a growth medium, carbon skeletons of various types are also being assimilated from all pathways by cellular biosynthesis.

To determine the nature of secondary pathways involved, the radiorespirometric utilization of acetate and pyruvate was studied (Fig. 1). This organism readily utilized both substrates, as evidenced by the appearance of C^{14} in respiratory CO_2 soon after administration of the substrates. With acetate, C-1 was converted to CO_2 at a rate much faster than C-2. Previous manometric studies demonstrated oxidation of various intermediates of the TCA cycle by resting cells (7). Thus, it appears that the TCA cycle is operating for the utilization of acetate for respiratory and biosynthetic functions. With pyruvate as a substrate, it was found that its carboxyl carbon was rapidly decarboxylated, presumably giving rise to the formation of acetate, which in

turn was catabolized by way of the TCA cycle as evidenced by the preferential oxidation of C-2 of pyruvate (equivalent to C-1 of acetate) to CO_2 as compared with C-3 of pyruvate (equivalent to C-2 of acetate).

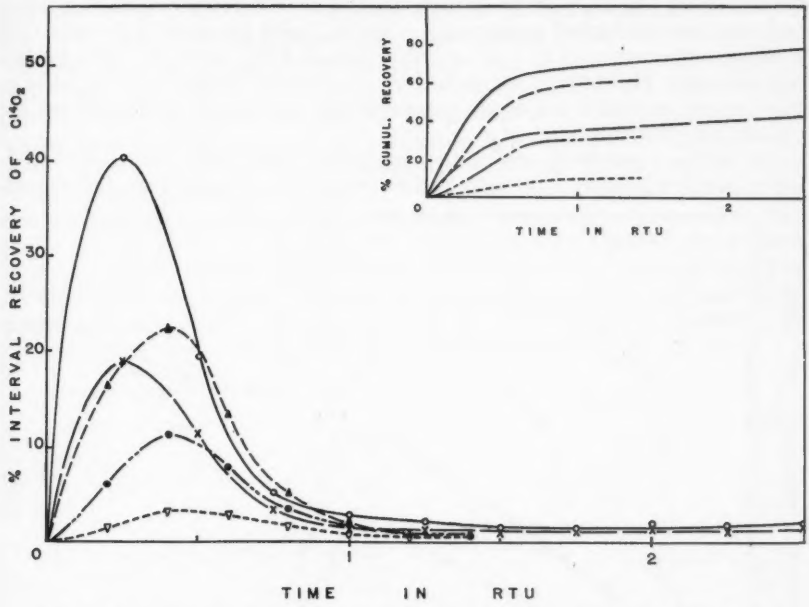

FIG. 1. Radiorespirometric pattern of interval and cumulative recoveries from *Micrococcus radiodurans* metabolizing specifically labelled acetate and pyruvate: ○ ——— sodium acetate-1-C^{14}; × — — sodium acetate-2-C^{14}; ▲ – – – – sodium pyruvate-1-C^{14}; ● – - - – sodium pyruvate-2-C^{14}; ▽ - - - - - sodium pyruvate-3-C^{14}.

NOTE: RTU = relative time unit. A relative time unit is defined as the time required in a given experiment for an organism to consume all of the intact labelled substrate originally added, as indicated in this case by the levelling of cumulative $C^{14}O_2$ recovery from glucose-3,(4) (8).

The operation of the TCA cycle for the biosynthetic functions is further supported by the observed incorporation of the substrate into the cellular constituents. As indicated in Table I, C-3 and C-2 of pyruvate were more heavily

TABLE I

Radiochemical recoveries from *Micrococcus radiodurans* metabolizing C^{14} specifically labelled acetate and pyruvate

Substrate, 2.5 mg	Radioactivity, c.p.m.	Per cent radiochemical recoveries		
		CO_2	Medium	Cells
1. Acetate-1-C^{14}	85,128	78	4	25
2. Acetate-2-C^{14}	59,238	43	7	56
3. Pyruvate-1-C^{14}	100,323	61	35	12
4. Pyruvate-2-C^{14}	99,379	32	36	26
5. Pyruvate-3-C^{14}	107,397	10	60	24

incorporated into the cells than C-1 of pyruvate and, similarly, C-2 of acetate was more heavily incorporated than C-1 of acetate. These findings are in accordance with the conservation of methyl carbon of either acetate or pyruvate when the TCA cycle is instrumental in the biosynthesis of various carbon skeletons for cell construction (8). It is also interesting to note that a significant amount of C-1 of pyruvate (about 12%) was also incorporated into the cells. This finding suggests that CO_2 fixation of the C_3+C_1 type may be functioning in this organism to provide the all-important C_4 skeleton for cellular biosynthesis.

The primary catabolic pathways functioning were studied using C^{14} specifically labelled glucose as substrates in a series of radiorespirometric experi-

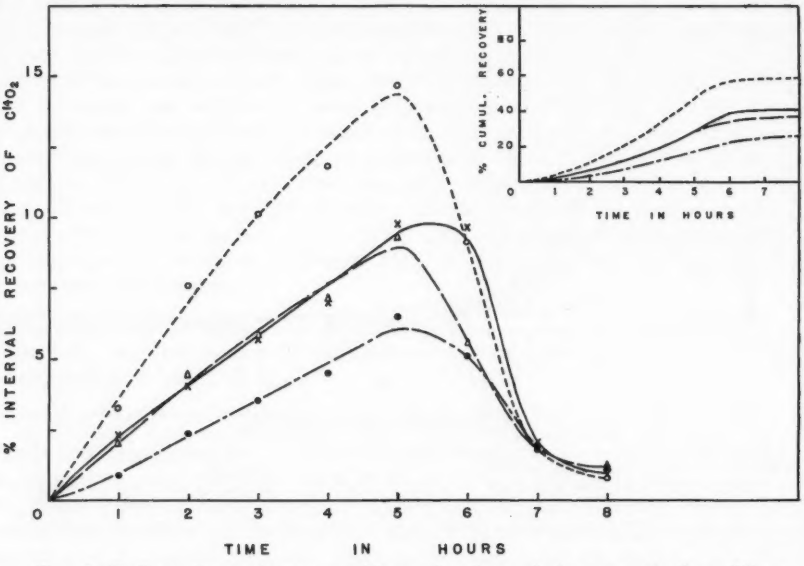

FIG. 2. Radiorespirometric pattern of interval and cumulative recoveries from *Micrococcus radiodurans* metabolizing specifically labelled glucose: × ——— D-glucose-1-C^{14}; △ — — D-glucose-2-C^{14}; ○ - - - - - - D-glucose-3,(4)-C^{14}; ● — - — - D-glucose-6-C^{14}.

TABLE II

Radiochemical recoveries from *Micrococcus radiodurans* metabolizing C^{14} specifically labelled glucose

Substrate, 7.5 mg/15 ml	Radioactivity, c.p.m.	Per cent radiochemical recoveries			
		CO_2	Medium	Cells,* approx.	Total
Glucose-1-C^{14}	120,607	42	17	40	99
Glucose-2-C^{14}	118,570	37.5	17	49	103.5
Glucose-3(4)-C^{14}	53,735	59.5	13.5	25	98
Glucose-6-C^{14}	103,313	26	19	53	98

*Calculated on the basis of 40 mg cells (dry weight) per flask.

ments. It was noted that C-3,(4) were converted to CO_2 in preference to any other carbon atoms of glucose as shown in Fig. 2 and Table II. Judging by the rapid rate and the total extent of its conversion (the final radiochemical recovery being about 60%), it appears that the bulk of the administered glucose was utilized by the Embden–Meyerhof–Parnas (EMP) glycolytic pathway, followed by the decarboxylation of pyruvate to yield acetate, which in turn was metabolized by way of the TCA cycle. This conclusion was also supported by the fact that C-2 of glucose (equivalent to acetate carboxyl carbon according to the EMP scheme) was preferentially oxidized to CO_2 as compared with C-6 of glucose, which was equivalent to acetate methyl carbon. Since C-1 and C-6 of glucose are equivalent in the EMP–TCA scheme, the observation that oxidation of C-1 to CO_2 was considerably more pronounced than that of C-6 reflects the occurrence of an alternative pathway in glucose catabolism. In the absence of enzymic studies it was difficult to define the exact nature of the alternative mechanism. However, it appears that a C_1–C_5 cleavage mechanism of the phosphogluconate decarboxylation (PGD) type was operative (4).

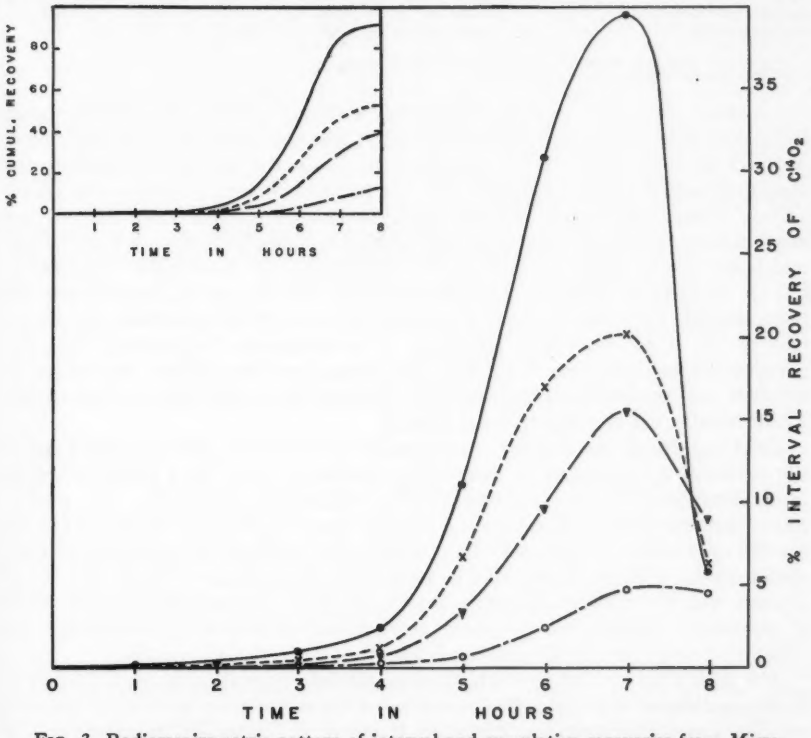

FIG. 3. Radiorespirometric pattern of interval and cumulative recoveries from *Micrococcus radiodurans* metabolizing specifically labelled gluconate (in the presence of glucose): ● ——— sodium gluconate-1-C^{14}; ▼ — — potassium gluconate-2-C^{14}; × - - - - - potassium gluconate-3,(4)-C^{14}; ○ — - — - potassium gluconate-6-C^{14}.

In order to test this possibility, C^{14} specifically labelled gluconate was used in combination with ordinary glucose as co-substrates in a series of radiorespirometric experiments. The pattern obtained is given in Fig. 3. Following an initial lag phase the administered gluconate was readily utilized as indicated by the recovery of considerable amounts of $C^{14}O_2$ from the labelled substrate (Table III). The $C^{14}O_2$ from C-1 was notably greater than that from any other

TABLE III

Radiochemical recoveries from *Micrococcus radiodurans* metabolizing C^{14} specifically labelled gluconate

Substrate*	Amount, mg/15 ml	Radio-activity, c.p.m.	Per cent radiochemical recoveries		
			CO_2	Medium	Cells, approx.
Na-gluconate-1-C^{14} +K-gluconate (unlabelled)	(negligible) 4.9	22,022	93	8	3
K-gluconate-2-C^{14}	6.6	22,047	39	8	44
K-gluconate-3,4-C^{14}	4.9	17,660	53	10	48
K-gluconate-6-C^{14}	6.6	21,243	14	13	74†

*In addition, each flask contained 3.75 mg unlabelled D-glucose.
†Determined by direct count of dry cells.

C atoms of gluconate; in fact, an over-all recovery of 93% was registered at the end of the experiment. This observation was consistent with the operation of PGD as the initial step in catabolism of gluconate. Much information can also be obtained by examining the relative rates of conversion of the remaining carbon atoms of gluconate to CO_2. As shown in Fig. 3, the order of the relative rates of conversion to CO_2 was C-3,(4) (52%) > C-2 (39%) and > C-6 (13%), which is in sharp contrast to the order observed with *Acetobacter suboxydans*, C-2 > C-3,(4) > C-6, an organism reported (5) to rely exclusively on the pentose cycle for its catabolism of glucose. In view of the demonstrated operation of the EMP glycolytic pathway, it is reasonable to conclude that the pentose, as derived from gluconate, may have been routed by way of transketolase and transaldolase reactions to fructose (2), which was in turn catabolized mainly via the glycolytic pathway.

Data regarding the cellular incorporation of C^{14} from glucose also suggest the probable operation of an alternative pathway involving a C_1–C_5 cleavage mechanism in addition to the EMP–TCA pathway (Table II). The preferential incorporation of C-6 into the cellular constituents can be accounted for by the operation of the EMP–TCA sequential pathway in providing carbon skeletons for cellular biosynthesis. However, as much as 25% of C-3,(4) of glucose was detected in the cells, arising perhaps through the incorporation of pentose (derived from glucose) into cellular nucleic acid, or through the utilization of pyruvate (originated from glucose) by a CO_2 fixation.

The pathway for the over-all glucose catabolism was estimated according to the method of Wang (8). Bearing in mind the assumptions made in the work on *Saccharomyces cereviseae* (8), and ignoring the possible incorporation of glucose into cellular polysaccharide, it was calculated on the basis of the radiorespirometric data collected at the end of 1 RTU (in this case, 7 hours) that

approximately 16%[4] of administered glucose was catabolized via the C_1–C_6 cleavage pathway. The bulk of the glucose appears to be utilized by way of the EMP pathway in conjunction with the TCA cyclic processes.

However, it is equally possible that the administered glucose may have undergone catabolism in the manner similar to that of the Entner–Doudoroff pathway reported for the pseudomonads. Further work is needed to clarify this point.

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[4]The fraction of glucose catabolized via a C_1–C_6 cleavage pathway, designated as C_p, was calculated by the following equation derived from that of Wang (8): $C_p = C_1'' - C_6''$, where C_1'' and C_6'' = fraction of the total activity administered as glucose-1-C^{14}, or -6-C^{14}, respectively, which was recovered in metabolic CO_2 up to the time of 1 RTU, from cells utilizing equal chemical amounts of glucose.

At the end of 1 RTU (7 hours), $C_1'' = 0.41$, $C_6'' = 0.25$, and $C_p = 0.41 - 0.25 = 0.16$.

INFLUENCE OF INSECT LURES ON HYPHAL GROWTH AND SPORULATION OF CHOANEPHORA TRISPORA[1]

JAMES E. ZAJIC[2] AND H. H. KUEHN[3]

Abstract

The influence of 10 insect lures on hyphal growth and asexual and sexual sporulation of the heterothallic fungus *Choanephora trispora* was determined. The least toxic lures were 1,2-hexadecanediol, 1,2-epoxy-hexadecane, and Gyplure, while Trimedlure, Medlure, and methyl eugenol were the most inhibitory to hyphal growth. In general, if an insect lure permitted hyphal growth, formation of some sporangia was observed. Under normal conditions the plus mating type formed more sporangia than did the minus strain. Sporangia formation of both mating types was stimulated by 1,2-hexadecanediol. Methyl eugenol (0.06%) was observed to disrupt and inhibit the positive chemotactic response of the hyphae for the opposite mating types which is observed prior to formation of zygospores. In addition, two other eugenol derivatives, 0.03% eugenol acetate and 0.003% isoeugenol formate, were found to inhibit inception of zygospores. Levels of eugenol which did not influence hyphal growth also did not inhibit carotene synthesis, which is interrelated with the sexual processes of this fungus. Isoeugenol formate partially inhibited sporangia formation in both mating types.

Introduction

A number of chemical insect attractants have been developed in recent years and their effectiveness evaluated in increasing the efficiency of insecticides (3). With the exception of studies on insects no evaluation has been made of the effect of insect lures on the micro- and macro-fauna found in nature. Of special interest is the fact that some of these insect lures appear to be highly specific sex attractants. An example of such a lure is acetoxy-hexadecen-1-ol, a natural sex attractant isolated from the female gypsy moth. From a comparative biochemical viewpoint the function of some natural insect lures as highly potent sex attractants may exist for the mating types of certain heterothallic fungi.

Comparatively little is known about the influence of insect lures on microbes. The following study was made to determine the effect of insect lures on hyphal growth, and on asexual and sexual fruiting of the heterothallic fungus *Choanephora trispora.*

Materials and Methods

Strains NRRL A-9216 (plus) and A-9159 (minus) of *C. trispora* were obtained from Dr. C. W. Hesseltine, USDA Northern Utilization Research and Development Division, Peoria, Illinois. These strains were grown separately at 28° C on slants of commercial potato-dextrose agar (PDA) at pH 7.0. The insect lures were provided by Dr. M. Beroza, USDA Agricultural Research Service, Beltsville, Maryland.

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The following procedure was used to determine the chemotactic or attractive ability of the insect lures for hyphae of *C. trispora*. Ten-milliliter aliquots of PDA were used per Petri dish. Sterile, highly absorbent cellulose disks 12.7 mm in diameter, commonly used in antibiotic assays, were placed aseptically on the agar surface. Quantitative amounts of up to 300 μg of the lures to be tested were added to the disks by means of a pipette. Sporangia of each mating type were inoculated onto each plate to form a 5-cm triangle in relation to the paper disk. The chemotactic influence was determined by measuring the distance of the margin of the colony from the disk after 24, 48, and 72 hours of incubation.

A second method similar to the one previously described, but not to be confused with it, was used to determine the chemical effect of each lure on hyphal growth and on the formation of sporangia and zygospores. For this determination specific amounts of each lure were added directly to 10 ml of PDA in Petri dishes. Hyphal growth was determined by measuring the size of the colony at various time intervals. The number of sporangia formed by each mating type was determined by placing a sterile 12.7-mm-diameter antibiotic disk (area = 1.27 cm^2) next to the original inoculum. With the aid of a binocular dissecting microscope, the number of sporangia per 1.27 cm^2 was counted. The paper disks alone did not inhibit hyphal growth or sporangia formation. Excellent contrast was observed between the black sporangia and the white disks. Quantitative determination of zygospores was much more difficult. A procedure was adapted, similar to that described by Hepden and Hawker (5), for measuring zygospore formation by homothallic strains of *Rhizopus sexualis*. Zygospores were counted under a binocular dissecting microscope at 375× magnification and the average number of zygospores formed in four microscopic fields determined.

The influence of eugenol derivatives on carotene synthesis was measured using methods described by Anderson (1).

Results

Of the 10 insect lures, only Gyplure appeared to show positive chemotactic activity under the conditions tested. Accelerated growth of both plus and minus mating types toward the disks containing Gyplure was slightly greater than on the controls.

Determination was made of the ability of strains A-9216 and A-9159 of *C. trispora* to grow on PDA in the presence of 0.3%, 0.03%, and 0.003% of the 10 insect lures indicated in Table I. Following 72 hours' incubation the diameter of the colonies was measured. Results (Table I) showed that three lures, 1,2-hexadecanediol, 1,2-epoxy-hexadecane, and Gyplure, were nontoxic at 0.3%, which was the highest level tested. Hyphal growth of both strains was slightly inhibited by 0.3% 4-(*p*-hydroxyphenyl)-2-butanone and completely inhibited by 0.3% of either anisylacetone, Siglure, Medlure, Trimedlure, Cuelure, or methyl eugenol. When the 10 lures were tested at a level of 0.03%, Trimedlure was the most inhibitory to hyphal growth, while Medlure and methyl eugenol were the most inhibitory at a concentration of 0.003%.

TABLE I

Influence of insect lures on hyphal growth and sporangia formation in NRRL A-9216(+) and A-9159(−) (*Choanephora trispora*) following incubation for 72 hours at 28° C

Insect lure	Concn. (%)	Colony diameter (mm)		Sporangia/1.27 cm²	
		A-9216	A-9159	A-9216	A-9159
1,2-Hexadecanediol	0.3	86	86	212	192
	0.03	86	86	342	136
	0.003	86	86	102	84
Anisylacetone	0.3	0	0	0	0
	0.03	54	62	0	15
	0.003	86	86	228	92
Siglure	0.3	0	0	0	0
	0.03	86	86	152	162
	0.003	86	86	154	92
1,2-Epoxyhexadecane	0.3	86	86	2	26
	0.03	86	86	15	39
	0.003	86	86	224	58
Medlure	0.3	0	0	0	0
	0.03	12	14	0	0
	0.003	77	77	30	200
Trimedlure	0.3	0	0	0	0
	0.03	0	0	0	0
	0.003	86	86	132	60
4-(*p*-Hydroxyphenyl)-2-butanone	0.3	62	64	20	2
	0.03	86	86	216	134
	0.003	86	86	240	124
Cuelure	0.3	0	0	0	0
	0.03	68	69	18	26
	0.003	86	86	220	80
Gyplure	0.3	86	86	120	21
	0.03	86	86	216	88
	0.003	86	86	224	108
Methyl eugenol	0.3	0	0	0	0
	0.03	33	30	0	0
	0.003	76	76	68	132
Controls		86	86	232	62

Under normal conditions the plus strain of *C. trispora* formed more sporangia than the minus strain. Although a larger variation in sporangial formation was observed in replicate plates, the ratio of sporangia formed by plus and minus strains was about 4:1. In these experiments, if an insect lure permitted hyphal growth, usually some asexual sporulation was observed. In most instances the plus strain formed more sporangia than the minus strain. However, the minus strain formed more sporangia than the plus strain in the presence of 0.003% Medlure. A level of 0.03% 1,2-hexadecanediol stimulated an average of about 100 more sporangia per 1.27 cm² than the plus strain controls and 70 more than the minus strain controls.

Probably the most interesting observation made in the experiments in which the insect lures were added directly to the plating medium was in regard to the influence of zygospore formation. Especially noteworthy were the results obtained with methyl eugenol. Addition of 0.03% and 0.003% methyl eugenol to the plating medium partially or completely inhibited the initial stage of zygospore formation, as demonstrated in Fig. 1. Plates A and B were treated respectively with 0.03% and 0.003% methyl eugenol while plate C is the control. Plates were inoculated adjacent to the antibiotic disks (used for sporangia counts) with strain A-9216 on the left and A-9159 on the right. Plate C demonstrates normal conditions, showing the degree of hyphal surface growth in what is referred to here as the zone of zygospore formation. Although there is slightly less hyphal overgrowth in the zone of zygospore formation, a complete intermingling of the hyphae of the two mating types, as well as normal zygospore formation, was observed. The dark zygospores

TABLE II

Influence of eugenol and eugenol derivatives on hyphal growth and sporulation of *C. trispora* NRRL A-9216(+) and A-9159(−)

Compound	Concn. (%)	Colony diameter (mm)		Sporangia/1.27 cm²		Zygospores per field
		9216+	9159−	9216+	9159−	
Eugenol	0.06	0	0	0	0	0
	0.03	0	0	0	0	0
	0.006	86	86	152	31	74
	0.003	86	86	57	11	88
	0.001	86	86	128	2	124
Methyl eugenol	0.06	69	62	25	158	9
	0.03	84	83	200	27	2
	0.006	86	86	264	148	122
	0.003	86	86	249	14	254
	0.001	86	86	228	3	152
Eugenol acetate	0.06	0	0	0	0	0
	0.03	73	40	0	0	0
	0.006	86	86	25	10	188
	0.003	86	86	216	7	240
	0.001	86	86	158	3	80
Eugenol phenyl acetate	0.06	62	71	32	11	340
	0.03	74	77	48	5	202
	0.006	79	82	73	9	176
	0.003	86	86	38	1	534
	0.001	86	86	142	6	300
Isoeugenol	0.06	0	0	0	0	0
	0.03	0	0	0	0	0
	0.006	79	79	216	31	254
	0.003	86	86	51	9	340
	0.001	86	86	132	4	244
Isoeugenol formate	0.06	28	36	3	1	0
	0.03	37	37	6	1	0
	0.006	30	40	0	0	0
	0.003	50	45	9	4	0
	0.001	84	86	30	3	56
Controls		86	86	138	30	150

PLATE I

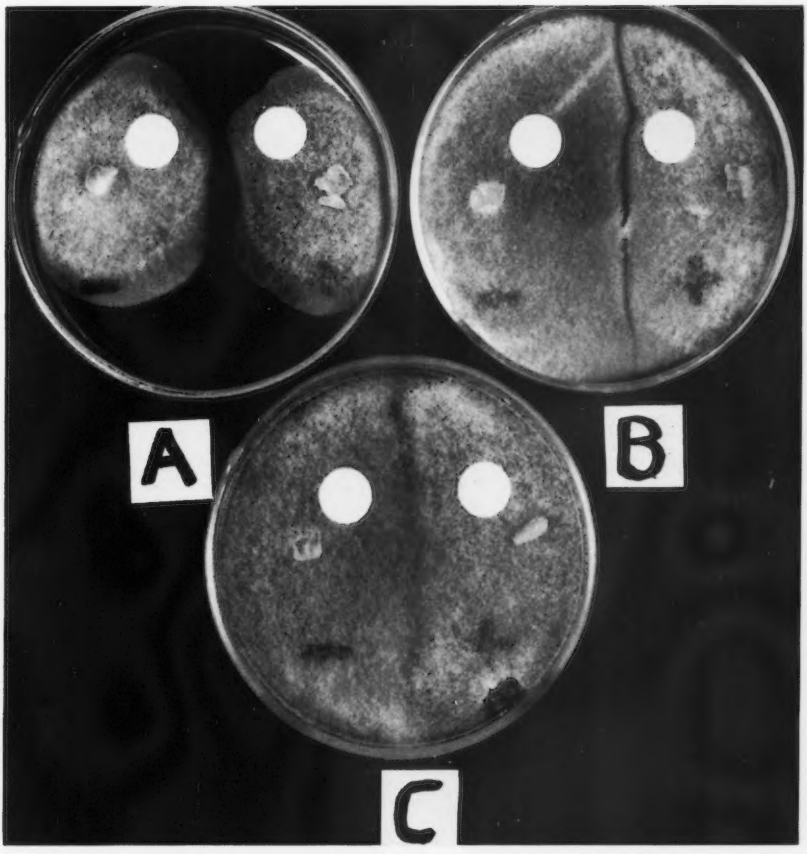

FIG. 1. The effect of methyl eugenol on hyphal growth in the zone of zygospore formation of strains A-9216 and A-9159 of *C. trispora*. A, 0.03% methyl eugenol; B, 0.003% methyl eugenol; C, control.

on plate C are hard to discern from the dark background, which was used to obtain a high contrast with the hyphae and to demonstrate the effect of methyl eugenol on hyphal growth in the "zygospore zone". In the presence of methyl eugenol not only was the rate of hyphal growth inhibited, but there was a strong inhibition of the positive chemotactic growth of hyphae of opposite mating types toward one another. Plates A and B show complete and partial inhibition of hyphal overgrowth in the zygospore zone. Contiguous growth was immediate under normal conditions. Complete or partial inhibition of zygospore formation was observed on addition of either 0.03% or 0.003% methyl eugenol. The zone of hyphal inhibition occurring in the "zygospore zone" extended up to a distance of 7 mm. Laterally growing hyphae, which reached the zygospore zone later than the faster growing peripheral hyphae, were less inhibited. At lower concentrations of methyl eugenol the inhibition was partially overcome, and it was followed by renewed growth and limited zygospore formation.

There is reported to be a mutual stimulation of the opposite strains of the heterothallic Mucorales due to complementary hormones (4). The disruption of this complementary relation by methyl eugenol stimulated an investigation to ascertain what other derivatives related to methyl eugenol might inhibit zygospore formation. Six eugenol derivatives were tested, and results showed (Table II) that 0.03% eugenol acetate and 0.003% isoeugenol formate completely prevented formation of zygospores, whereas 0.06% methyl eugenol was almost as effective. A slight initial inhibition of the normal positive chemotactic response resulted with 0.06% eugenol phenyl acetate, but normal contiguous hyphal growth soon followed.

Almost all of the eugenol derivatives inhibited hyphal growth at concentrations of 0.06%. Some growth was observed in the presence of 0.06% methyl eugenol and eugenol phenyl acetate, but these results may be misleading since at such high concentrations these compounds were not completely soluble and evenly dispersed in the medium. Eugenol and isoeugenol were the most toxic to growth. Sporangia were formed where hyphal growth was sufficient. Isoeugenyl formate partially inhibited formation of sporangia as well as zygospores even at the lowest level of 0.001%. The highest zygospore counts, ranging from 300 to 500 per field, were obtained with certain concentrations of eugenol phenyl acetate.

Concentrations of 0.006% of either eugenol or methyl eugenol, or 0.05%

TABLE III

The influence of eugenol on carotene synthesis by mixed cultures of *C. trispora* NRRL A-9216(+) and A-9159(−)

Compound	Concn. (%)	beta-Carotene (mg/100 ml medium)
Eugenol	0.09	5.9
	0.06	20.8
	0.03	52.6
	0.003	44.0
	0.0003	50.0
Control	0.0	45.2

eugenol phenyl acetate, permitted the greatest development of carotenoid pigmentation, as determined visually by the presence of a yellow zone in the area of zygospore formation. A more specific determination of the influence of these three compounds on carotene synthesis was made by using the optimal fermentation conditions described by Anderson (1) for mixed cultures of *C. trispora*. Since the results were similar for all three compounds, only the data for eugenol are presented in Table III. The addition of 0.06–0.09% eugenol to the fermentation medium inhibited carotenogenesis, giving yields of 20.8 mg per 100 ml medium or less of beta-carotene. In some instances lower concentrations of 0.03–0.0003% eugenol gave slight increases in carotene yields over the controls, which averaged 45.2 mg per 100 ml, but such increases were not regarded as significant.

Discussion

A number of factors have been reported to inhibit zygospore formation. Hawker (4) discussed the role of the C:N ratio and the importance of nitrogenous substances in this process. High concentrations of nitrate and asparagine have been reported to inhibit zygospore formation in *Phycomyces blakesleeanus* (4). Some natural inhibitors have also been reported, such as the inhibiting substances from maturing sclerotia which inhibit development of rhizomorphs of *Armillaria mellia* (4). However, this is the first time the authors have encountered specific chemicals, such as methyl eugenol, which inhibit zygospore formation.

At least two other derivatives of eugenol, 0.03% eugenol acetate and 0.003% isoeugenol formate, were active in inhibiting zygospore formation. Although a variety of extraspecific chemicals or hormones have been reported which regulate the sexual processes in fungi (8, 9), the interrelation of these hormones with morphological development has been worked out for only one or two fungi. If the sexual processes of *C. trispora* are similar to those of heterothallic species of *Achlya* (9), each culture may secrete one or more hormones which initiate, augment, or inhibit the initial phase of zygospore formation. Probably it is this initial phase of chemotropism between hyphae of opposite mating types that is inhibited in *C. trispora* by methyl eugenol. Complete inhibition of the initial phases of zygospore formation was associated with a subnormal amount of hyphal growth in an area 1–7 mm wide. With low concentrations of the effective eugenol derivatives, the inhibition of the chemotropic response was gradually overcome and limited formation of zygospores followed. In the presence of eugenol phenyl acetate counts of zygospores were the highest obtained anywhere in this study.

The relation of carotene biosynthesis to the sexual processes of the Choanephoraceae has been described by Barnett, Lilly, and Krause (2). Hesseltine and Anderson (6) also studied the relation of carotenogenesis to sexuality in Choanephoraceae. Recently, Hesseltine and Benjamin (7) have reported on the influence of nutrition and physical conditions on growth and sporulation of *C. trispora*. They found that high levels of glucose and temperatures of 32–37° C favored carotene biosynthesis. However, low concentrations of carbohydrates and a temperature of 25° C favored production of sporangia. In the

present study certain insect lures reduced the numbers of zygospores produced, while other lures stimulated zygospore formation. No significant stimulation of carotenogensis was correlated with the increase in the numbers of zygospores produced.

Gyplure may have potential sex-attractant activity for the mating types of *C. trispora*. Present methods are inadequate to measure this particular property. Under the conditions tested no toxicity was observed with three of the insect lures at a concentration of 0.3%: these were 1,2-hexadecanediol, 1,2-epoxyhexadecane, and Gyplure. Partial inhibition of hyphal growth was observed with 0.3% 4-(*p*-hydroxyphenyl)-2-butanone, whereas there was complete inhibition of hyphal growth with 0.3% of either anisyl acetone, Siglure, Medlure, Trimedlure, Cuelure, or methyl eugenol. The growth-inhibiting activity of the insect lures was much reduced at concentrations of 0.03–0.003%. The antimicrobial activity of some of these sex attractants for *C. trispora* indicates that there is need for further microbiological testing.

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DEGRADATION OF THE POLYSACCHARIDE OF XANTHOMONAS PHASEOLI BY AN EXTRACELLULAR BACTERIAL ENZYME[1]

S. M. LESLEY

Abstract

A species of *Bacillus* isolated from soil was found to elaborate an inducible, extracellular enzyme capable of degrading the polysaccharide of *Xanthomonas phaseoli* to low-molecular-weight oligosaccharides. The enzyme was characterized with respect to pH optimum, ion requirement, and stability. Examination of the polysaccharide degradation products revealed that the glucuronic acid moiety of the polysaccharide was altered to a $\Delta^{4,5}$ unsaturated form as a result of enzyme action.

Introduction

The extracellular polysaccharide of *Xanthomonas phaseoli*, agent of common blight of beans, has been purified and found to contain equal proportions of D-glucose, D-mannose, and D-glucuronic acid (8). An acid-resistant aldobiouronic acid type of linkage joins a large part of the glucuronic acid (GA) to mannose in this polysaccharide,[2] and, as a result, the acid-labile GA is largely destroyed during acid hydrolysis of this macromolecule. In a study of the biosynthesis of this polysaccharide, C^{14}-labelled material was used to trace the synthetic pathway. Since this technique involved an analysis of the distribution of the C^{14} label in each carbon atom of the three component hexoses, it was essential to find another means of obtaining a reasonable yield of the GA component.

In many investigations, dating from the work of Dubos in 1931 (4, 18) various enzymes have been employed to degrade specific polysaccharides to fragments of low molecular weight (13). Such small fragments have proved to be extremely useful in studies on the structure of the original polysaccharide since the classical methods of carbohydrate chemistry can be applied with more success to short-chain-length oligosaccharides than to the whole undegraded molecule (14). A search was made, therefore, for an enzymatic method of degrading this polysaccharide as an aid to both structural analysis and isolation of the GA component.

Methods

Reducing sugars were estimated by Nelson's colorimetric modification (16) of the micro-Somogyi method. Uronic acid was estimated by the carbazole – sulphuric acid method of Dische (3) with pure GA as a standard, while total carbohydrate concentration was determined by the indole – sulphuric acid method (3) or by oxidation to carbon dioxide by the modified persulphate method described by Katz *et al.* (7), with glucose as the standard.

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[2]Author's unpublished results.

For identification purposes, descending paper chromatography was employed using Whatman No. 1 filter paper. Where material was to be eluted for further study, Whatman No. 3 MM filter paper was used. A preliminary irrigation with distilled water was essential to remove from this paper material absorbing in the ultraviolet region. The developing solvents were (A) *n*-propanol – ethyl acetate – water (7:1:2) and (B) *n*-butanol – acetic acid – water (44:16:40). An apparatus similar to that described by Block, Durrum, and Zweig (2) was used for paper electrophoresis. The electrolyte was 0.05 *M* acetate buffer at pH 3.5 and the potential difference was 40 v per cm at a current of 20 ma. Chromatograms were sprayed with the *o*-aminobiphenyl reagent described by Timell *et al.* (21), and with the periodate reagent described by Metzenberg and Mitchell (15).

The polysaccharide of *X. phaseoli* was produced and purified as described previously (8).

Specific viscosity was measured with an Ostwald-type viscometer. Viscometers with two different sizes of capillary were used and, with each, the outflow time was first determined for the reference liquid (distilled water). In the standard test the mixture contained enzyme, 5 mg of polysaccharide, and 300 micromoles of tris (hydroxymethyl) aminomethane (TRIS) buffer in a volume of 6 ml at pH 6 in an Ostwald viscometer at 30° C. Samples required for other analyses were always removed from a separate incubation mixture of the same components incubated independently at the same temperature. One unit of enzyme activity was arbitrarily designated as that amount of enzyme required to produce a decrease in specific viscosity of 0.1 in 10 minutes under standard conditions.

It was known (1) that the rate of decrease in specific viscosity of hyaluronic acid solutions by hyaluronidase was markedly influenced by the initial viscosity. A similar effect was also found in this work with different preparations of polysaccharide. Particular care was taken to use the same preparation of polysaccharide for related experiments.

Experimental and Results

Several commercially available enzymes were tested (β-glucuronidase, α- and β-amylase) as well as a sample of a pure cellulase[3] but no degradation of the polysaccharide was detected with any of these preparations. A successful attempt was then made to isolate a soil bacterium capable of degrading this material. About 20 g of soil was enriched with 5 mg of pure polysaccharide and kept moist at 27° C. At daily intervals, an aliquot of this mixture was diluted with water and three volumes of alcohol were added to precipitate any undegraded polysaccharide. After 7 days' incubation this simple test indicated that polysaccharide degradation had occurred. Microscopic examination of the soil culture at this stage showed a large number of sporeforming bacteria. An aliquot of the mixture was heated at 70° C for 20 minutes to destroy all vegetative cells. This heated soil culture was tested as before, and polysaccharase activity was again found. Suitable dilutions of the heated soil were plated on Pennassay Seed Agar (Difco) and a representative colony

[3]A gift of Dr. D. R. Whitaker, National Research Council, Ottawa.

was isolated. The resulting pure culture was tested by growth in yeast extract broth medium containing sufficient polysaccharide to form a definite precipitate when an aliquot was mixed with three volumes of alcohol. The polysaccharide was found by this test to be actively degraded during growth of this organism. The bacterium was identified as a species of *Bacillus* by Dr. I. L. Stevenson of this Institute and was maintained as Bacillus SB II, on Pennassay Seed Agar slants.

Growth of the Organism and Enzyme Preparation

Preliminary experiments had indicated that the enzyme activity produced in these cultures was extracellular, and as an aid in subsequent purification of the enzyme the simplest medium suitable for growth of the organism was chosen. Some difficulty was encountered in preventing sporulation while obtaining good multiplication and enzyme production. By testing a variety of additives to a basic mineral salts solution the following medium was selected for the studies to be described below. Each liter of the medium contained the mineral salts described by Starr (19), modified to an increased buffer concentration of 0.03 *M* phosphate, as well as the following additives: 30 ml soil extract prepared by the method of Erwin and Katznelson (5), 0.8 g Difco brain heart liver dehydrated medium, 250 mg glucose, and 150 mg of the pure polysaccharide of *X. phaseoli*. The medium was adjusted to pH 7, dispensed in 600-ml amounts in Fernbach flasks, and autoclaved at 15 lb for 15 minutes.

A typical enzyme preparation was made by inoculating 600 ml of medium in a Fernbach flask from a stock slant of SB II and incubating at 27° C on a rotary shaker at 100 excursions per minute. The optical density (Spectronic "20" at 600 mμ) increased from about 0.05 to 0.8 in 16 hours, but degradation of the polysaccharide did not occur until 40 hours, when the optical density was 0.96. The culture was chilled and the cells removed by centrifugation at 12,000 *g* for 10 minutes. The supernatant was concentrated by lyophilization and resuspended to the required concentration in distilled water. This was dialyzed against several changes of distilled water at 5° C for 72 hours and then frozen and stored at −30° C for future work.

The enzyme was found to be entirely extracellular and inducible. All extracts made from the intact cells were devoid of activity. These tests included suitable controls which showed that the cell extracts did not inhibit the activity of this enzyme. Evidence that the enzyme activity was produced only in response to the presence of the polysaccharide was obtained by inoculating two media, one containing polysaccharide and the other 1% glucose in place of the polysaccharide, with an enzymatically active, liquid culture and incubating as usual. Activity was absent in the cell-free supernatant of the culture to which no polysaccharide had been added. The control culture was found to contain the usual enzymatic activity.

Characteristics of the Enzyme Preparation

The following experiment was done in order to ascertain the possible relationship between enzyme concentration and activity. Increasing volumes of the enzyme solution were tested for polysaccharase action under standard

conditions as described in "Methods". The change in specific viscosity with time was followed for each concentration and the activity was calculated from the results. With 0.1, 0.25, 0.5, and 1.0 ml of the enzyme preparation, the number of units per ml was found to be 1.7, 1.8, 2.2, and 2.5, respectively. It is clear that for a quantitative determination of enzyme activity this method must be restricted to experiments where enzyme concentration is kept constant.

The change in enzyme activity with change in pH was determined viscometrically at 30° C as before. A final volume of 6 ml contained 5 mg polysaccharide, 0.01 *M* Tris buffer, 0.01 *M* phosphate buffer, and 0.5 ml of stock enzyme preparation. The pH of each test solution was adjusted to the desired value with dilute acid or alkali before the enzyme was added and was checked after the rate of decrease in specific viscosity had been determined for each solution. The results, presented in Fig. 1, indicate an optimum between pH 6 and 7.

The activity of a crude enzyme preparation was found to be markedly increased by dialysis against distilled water. A typical cell-free culture supernatant containing enzyme activity was concentrated 10 times by lyophilization. Equivalent aliquots of this preparation were tested before and after dialysis, and the number of units per milliliter was found to have been increased from 0.3 to 1. This increase in activity could be due to a reduction in the ionic concentration in the test system used.

There is evidently no ion requirement for activity of this enzyme preparation since the addition of neutralized ethylenediamine tetraacetic acid (1.5×10^{-4} *M*), cupric sulphate (10^{-3} *M*), or magnesium sulphate (10^{-3} *M*) had no effect on the rate of decrease of specific viscosity of the polysaccharide when a dialyzed enzyme preparation was used in the test.

Dialyzed enzyme preparations were very stable, especially when frozen and stored at −30° C. One preparation contained 1.2 units of activity per ml before freezing; after 25 months' storage at −30° C the preparation was retested under the same conditions and found to contain the same activity.

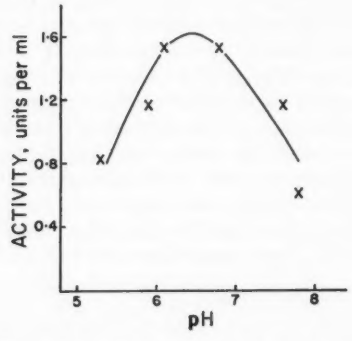

FIG. 1. Effect of pH on the activity of the enzyme preparation. The arbitrary units of activity were determined as described in "Methods".

Enzyme activity was stable also at higher temperatures. Several dialyzed preparations were concentrated five times by evaporation in the Craig evaporator at a bath temperature of 40° C under reduced pressure with no loss of activity.

A close relationship was found to exist between the decrease in specific viscosity and the release of new reducing groups, due to the rupture of glycosidic bonds, during the enzymatic degradation of the polysaccharide. This can be seen in the data presented in Fig. 2, where curve 1 represents the results obtained from viscosity measurements and curve 2 the results from the determination of the increase in reducing groups, with time, in aliquots removed from a separate incubation mixture under identical conditions. Glucose was used as the standard in the latter method.

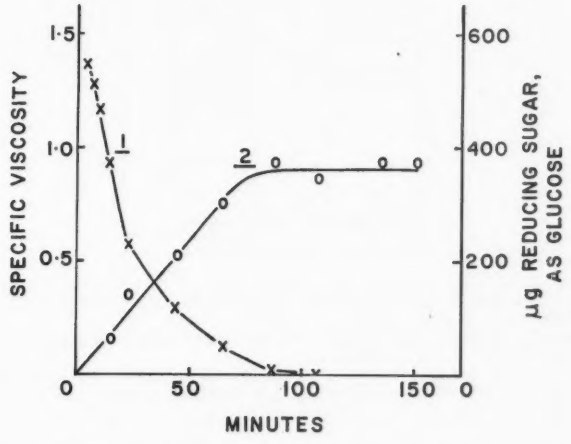

FIG. 2. Decrease in specific viscosity (curve 1) and release of reducing groups (curve 2) during degradation of *X. phaseoli* polysaccharide by a bacterial enzyme preparation.

Alteration of the Glucuronic Acid Component of the Polysaccharide

The products of the enzymatic degradation of the polysaccharide were examined by paper chromatography in solvent system B, and seven distinct bands were identified when the chromatogram was sprayed with either the *o*-amino-biphenyl reagent or the periodate reagent. Two areas showed absorption in the ultraviolet region when examined with a light source whose maximum emission was at 2570 Å. Each of the seven areas was eluted, hydrolyzed with 1 *N* sulphuric acid at 120° C for 60 minutes, neutralized with barium hydroxide, concentrated by evaporation, and chromatographed in solvent system A. Glucose and mannose were located on the chromatogram of each hydrolyzate by spraying with the *o*-amino-biphenyl reagent but glucuronic acid was not detected on any of the seven chromatograms. Similarly GA was not detected on a chromatogram of an acid hydrolyzate of an aliquot of the whole enzyme digest. It is noteworthy that the acid hydrolyzate of one area

(Fig. 3A, band 6) contained no glucose or GA but did contain mannose and a faster-moving unidentified material.

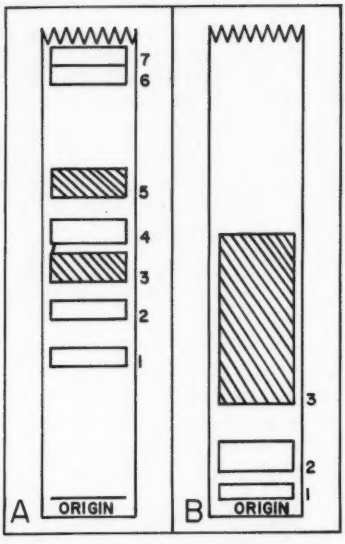

FIG. 3. Schematic representation of a paper chromatogram showing separation of the products of enzymatic digestion of the polysaccharide; the shading indicates absorption in the ultraviolet region.

A. Bands 1–7 represent components of an enzymatic digest of *X. phaseoli* polysaccharide as separated in solvent system C.

B. Bands 1–3 represent components of A, band 5, separated in solvent system A. Band 3 is a trisaccharide of $\Delta^{4,5}$ glucuronic acid, mannose, and glucose and is referred to in the text as component *5a*.

It appeared possible that the GA component had been converted to the $\Delta^{4,5}$ unsaturated form, which shows absorption in the ultraviolet region, as described by Linker *et al.* (9). Accordingly, an enzymatic digest was prepared with 1.94 g of purified polysaccharide and, after concentration by evaporation *in vacuo* to a minimum volume, the material was applied to large sheets of Whatman 3 MM filter paper. The components of the digest were separated by two excursions of 18 hours each in solvent system B. Strips were cut from the edges of the chromatograms and were examined by ultraviolet light and by spraying with the *o*-amino-biphenyl reagent. A schematic tracing of a typical chromatogram is shown in Fig. 3A. Band numbers 3 and 5 showed absorption in the ultraviolet region. Band 5 appeared to be the more concentrated component and it was eluted for further examination. This eluate was rechromatographed, with solvent system A, and the result is given as Fig. 3B, where it can be seen that only area number 3 showed absorption in the ultraviolet region. This portion was subsequently eluted and termed "digest component *5a*". An aliquot of this component was tested by paper electrophoresis at pH 3.5. Since only one area was found, it was judged to be chro-

matographically pure.

The absorption spectrum of component 5*a* at both acid and neutral pH was determined with a Beckman DK1 recording spectrophotometer. Tracings of these spectra are presented in Fig. 4. The change in the spectrum with pH is reversible and is characteristic of component 3 as well as of component 5, Fig. 3A.

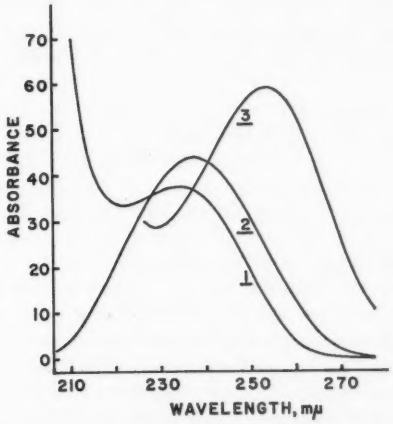

FIG. 4. Spectra of component 5*a* isolated from enzymatically degraded *X. phaseoli* polysaccharide: curve 1 at pH 1.5, and curve 2 at pH 7; curve 3 is the spectrum of a product isolated by paper chromatography from the acid hydrolyzate of component 5*a*.

The optical density of a solution of component 5*a* at pH 1.3 was determined at 237 mμ. The $\Delta^{4,5}$ GA content of this solution was determined with the carbazole – sulphuric acid reagent (3), with pure GA as the standard. The molar extinction coefficient was calculated, on the basis of the $\Delta^{4,5}$ GA content, to be $\epsilon_{237} = 6.35 \times 10^3$ liter mole^{-1}cm^{-1}.

The total carbohydrate content of the same solution of 5*a* was determined with the indole – sulphuric acid reagent (3), and this was checked by combustion of an aliquot of the same solution to carbon dioxide with the persulphate reagent (7). Glucose was used as the standard in both methods. The molar extinction coefficient was calculated on the basis of the total carbohydrate present and on the assumption that component 5*a* was a trisaccharide containing $\Delta^{4,5}$ GA, glucose, and mannose. The values thus obtained were: $\epsilon_{237} = 5.3 \times 10^3$ liter mole^{-1}cm^{-1} when the indole – sulphuric acid reagent was used and $\epsilon_{237} = 6 \times 10^3$ liter mole^{-1}cm^{-1} when the persulphate reagent was used. Both values are in reasonable agreement with that determined above on the basis of the $\Delta^{4,5}$ GA content alone, and therefore the assumption that 5*a* was a trisaccharide was taken as most probably correct.

An aliquot of component 5*a* was hydrolyzed with sulphuric acid and the hydrolyzate examined by paper chromatography in solvent system A. Only glucose and mannose were detected with the two spray reagents but a third

area was located by examination with ultraviolet light. This was eluted and its spectrum was determined, at pH 1.5. A tracing of this record is presented in Fig. 4, curve 3. This material has not been identified but is probably derived from component 5*a* of the enzymatic digest. Evidence for this was obtained as follows. *X. phaseoli* polysaccharide, produced during growth on universally C^{14}-labelled glucose, was degraded enzymatically and component 5*a* isolated as before, hydrolyzed, and the hydrolyzate chromatographed in solvent system A. The amount of C^{14} present in the glucose and mannose regions was 59 and 51 counts per minute, respectively, while the ultraviolet absorbing region contained 46 counts per minute. This material was not examined further.

Component 5*a*, as isolated above from the enzymatic digest of the polysaccharide, was subjected to ozonolysis, and oxalic acid was isolated from the resulting solution using the methods described by Linker *et al.* (9). The melting point of the isolated oxalic acid was 182° C whereas a sample of authentic oxalic acid melted sharply at 194° C (literature = 189° C (6)). A mixture of equal parts of authentic and isolated oxalic acid also melted sharply at 194° C. All melting points were uncorrected.

A small aliquot of the isolated oxalic acid was chromatographed with reference compounds including the four possible products that could result from ozonolysis of an unsaturated glucuronic acid type of molecule. These included maleic, oxalic, citric, and succinic acids. The acid solvent system and new spray reagent described by Paskova and Munk (17) were used for the chromatography of these acids. The newly isolated compound gave the characteristic shape, color reaction, and R_f of known oxalic acid. The isolation of oxalic acid supplied additional evidence that component 5*a* contained $\Delta^{4,5}$ unsaturated glucuronic acid.

Figure 5 shows the results of an experiment in which the enzymatic activity was followed by measuring the decrease in specific viscosity, while the formation of unsaturated GA was determined by measuring the increase in optical density at 237 mμ. The viscosity was measured in an Ostwald viscometer as detailed in "Methods", while aliquots were removed from a separate incubation mixture and diluted 1:4 in 0.01 *N* hydrochloric acid for the optical density measurement. It can be seen that the initial rapid decrease in specific viscosity of the polysaccharide solution was reflected in the initial rapid rise in optical density at 237 mμ. Thus it may be concluded that the rate of formation of the unsaturated GA is closely related to the decrease in specific viscosity during enzymatic degradation.

An attempt to fractionate the enzyme preparation was made in order to obtain an active preparation which would not alter the GA moiety of the polysaccharide. Ammonium sulphate fractionation of a stock enzyme preparation gave no precipitate below 0.65 saturation. The precipitate obtained at 0.65 saturation was collected, dialyzed, and tested against the polysaccharide. Chromatographic analysis of the digest in solvent system B resulted in a chromatogram identical with that seen previously (Fig. 3A) to result from the action of the original enzyme preparation. No fractionation of the two activities had been achieved.

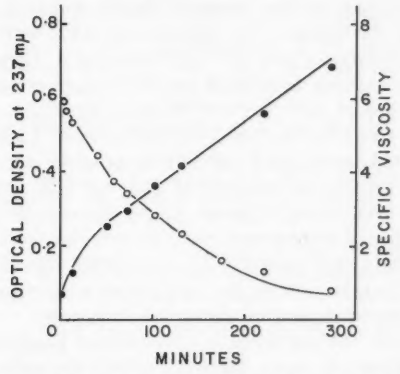

FIG. 5. Decrease in specific viscosity and increase in optical density at 237 mμ of *X. phaseoli* polysaccharide during degradation by a bacterial enzyme preparation.

In a later experiment the enzyme preparation was heated for 10 minutes at 53° C. This treatment destroyed the enzyme activity responsible for altering the GA component of the polysaccharide. In addition the rate of action of the heated enzyme, measured viscometrically, was significantly lowered. Furthermore the specific viscosity of the polysaccharide substrate was not reduced to zero, as it was by the original enzyme preparation, but levelled off well above this point. Moreover, on chromatographic examination of the digest it was found that only three areas could be separated in solvent system B and the major component remained near the origin. These results indicated that the extent of polysaccharide degradation was not as great with the heated enzyme as it was with the unheated enzyme preparation. A digest made with the heated enzyme was acid-hydrolyzed and the hydrolyzate examined by chromatography in solvent system A. The chromatogram showed the presence of GA, glucose, mannose, and the aldobiouronic acid always found an acid hydrolysis of the original polysaccharide. It was apparent that the enzymatic activity remaining in the heated enzyme had not destroyed the aldobiouronic acid type linkage between glucuronic acid and mannose. Clearly, therefore, the yield of GA obtained by acid hydrolysis of a heated enzyme digest would be no greater than that obtained by acid hydrolysis of the original intact polysaccharide.

Discussion

The enzyme preparation obtained from a species of *Bacillus* isolated from soil may be useful in obtaining information on the structural details of the polysaccharide of *X. phaseoli*. The fact that the enzyme activity can be altered by heating to give different and probably larger fragments of polysaccharides should be of additional value for this type of analysis. The double bond in the glucuronic acid should not be a hindrance since it may be saturated by a mild reduction procedure as shown by Linker *et al.* (9).

The action of the present enzyme preparation is apparently quite similar

in some respects to that of the bacterial hyaluronidase studied by Linker *et al.* (9), and later by other investigators (10, 12, 20). The trisaccharide containing glucose, mannose, and $\Delta^{4,5}$ GA found in a digest of bacterial polysaccharide with the enzyme described in this paper is comparable with the disaccharide, composed of $\Delta^{4,5}$ GA and N-acetylglucosamine, which is found in a digest of hyaluronate by bacterial hyaluronidase. This disaccharide is the repeating unit of hyaluronic acid, and it is possible that the trisaccharide studied here is an important structural unit of the polysaccharide of *X. phaseoli*. The other components shown to be present in the enzyme digest may well be the result of the second enzyme activity found to be present by the experiments with heated enzyme. It is possible, therefore, that the action of a highly purified fraction of this bacterial enzyme preparation would throw still more light on the structure of the polysaccharide.

From their study on the bacterial hyaluronidase Ludoweig *et al.* (10) have concluded that it shows a very unusual action in splitting the glycosidic linkage on the alcohol side of the oxygen bridge between C_1 of N-acetylglucosamine and C_4 of GA. Since the identical alteration of GA occurs in this enzyme–substrate system, the same type of elimination reaction also must occur. Suzuki (20) found that a bacterial chondroitinase preparation would act on chondroitin sulphates A and B and release $\Delta^{4,5}$ glucuronido-acetylgalactoseamine and its sulphate esters. It is possible that this type of reaction is not unusual and that, particularly in soil, where bacterial and plant polysaccharides containing GA are found in abundance (11), it may represent a major means of polysaccharide degradation.

Acknowledgment

I wish to express my appreciation to Dr. R. M. Hochster for his helpful advice during the course of this work, and to Dr. H. Katznelson for his aid in the preparation of the manuscript. I wish also to thank Mrs. Jean Ross for her expert technical assistance.

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THE DEVELOPMENT OF TIPULA IRIDESCENT VIRUS IN THE CRANE FLY, TIPULA PALUDOSA MEIG., AND THE WAX MOTH, GALLERIA MELLONELLA L.[1]

F. T. BIRD

Abstract

Patches of material rich in desoxyribonucleic acid form in the cytoplasm of cells of the silk gland, fat body, epidermis, tracheal epithelium, and muscles of the crane fly, *Tipula paludosa* Meig., and the wax moth, *Galleria mellonella* L., infected with *Tipula* iridescent virus. The development of virus particles appears to be associated with strands of dense material in the cytoplasm of susceptible cells. Each particle consists of a central core surrounded by a membrane. After their formation many of the particles appear to become surrounded by protein.

Larvae of the crane fly, *Tipula paludosa* Meig., are susceptible to a virus which, because of its peculiar optical properties, has been named *Tipula* iridescent virus. When the virus is concentrated by centrifuging it at 20,000 r.p.m., the resulting pellet shows an intense iridescence. An infected larva can be recognized by its violet coloration, which is caused by the optical effect of the microcrystals of virus in the tissues of the living insect (4).

The virus particles are comparatively large, measuring 130 mμ in diameter, and extremely uniform in size and shape. The shape of the virus particle has been shown to be an icosahedron (7). Each particle consists of a central core surrounded by two membranes in the form of two concentric hexagons (5). About 15% of the virus particle is desoxyribonucleic acid (DNA) (4).

According to Smith *et al.* (6) the initial site of multiplication of the virus is the cytoplasm of the fat body but, as the disease progresses, invasion of the epidermis and muscles occurs. The virus is found in enormous quantities in the infected insect and finally makes up about 25% of the dry weight of the larva (4).

Tipula iridescent virus has been successfully transmitted, by injection, to seven species of Diptera, eleven species of Lepidoptera, and three species of Coleoptera (6). Several larvae of *Pieris brassicae* L., infected with the virus, were received from Dr. K. Smith, Virus Research Unit, Cambridge, England, in October, 1960. The virus was purified and successfully transmitted to larvae of the wax moth, *Galleria mellonella* L., and to larvae of its original host, *Tipula paludosa* Meig. The following is a report of light and electron microscope studies on the development of the virus in these two insects and of some preliminary quantitative studies on virus dosage.

Methods

The virus was purified as follows. Larvae killed by the virus were macerated in distilled water and large particles removed by centrifuging at 200 *g* for 15 minutes. The supernatant was centrifuged at 13,000 *g* for 30 minutes.

[1]Manuscript received June 6, 1961.
Contribution No. 29, Insect Pathology Research Institute, Department of Forestry, Sault Ste. Marie, Ontario.

The resulting pellet was suspended in distilled water and the large particles removed by centrifuging at 200 *g* for 30 minutes. This procedure of alternating low- and high-speed centrifugations was repeated until a violet-green colored pellet was obtained. (The first pellets, which contained much extraneous material, were dark green in color.) The final pellet was suspended and stored at about 4° C in an antibiotic suspension[2] containing 20 μg of chloromycetin, 200 μg streptomycin, and 20 μg neomycin per ml of sterile distilled water. About 130 mg of the pure virus was obtained from 170 wax moth larvae.

Virus suspensions for injection into the larvae were prepared by diluting the stock suspension with sterile distilled water, and each larva was injected with 5 μl of the suspension containing from 7×10^{-6} to 7×10^{-13} g of protein.

Tissues for light-microscope studies were fixed in Bouin's fluid or in Gilson's fixative and embedded in paraffin wax. Sections cut at 4 μ were stained with Heidenhain's iron haematoxylin. The Feulgen method was used following fixation in Gilson's fixative to identify tissue containing DNA.

Tissues for electron microscopy were fixed in osmic acid buffered at pH 7.0. They were embedded in methacrylate and sectioned with a Sjöstrand ultramicrotome equipped with a diamond knife. An R.C.A. EMU-2 electron microscope was used.

Results

Since light-microscope studies have shown that the same symptoms of virus infection occur in both *T. paludosa* and *G. mellonella*, and since electron-microscope studies have shown a similar development of the virus in all susceptible tissues, the following description applies to both insects. The illustrations were selected only on the basis of their clarity in demonstrating some of the points discussed.

Light-microscope studies of sectioned and stained tissues of healthy larvae and of larvae taken at 24-hour intervals after injection with virus show that irregular patches of material, which stain intensely with iron haematoxylin, form in the cytoplasm of cells of the silk gland, fat body, epidermis, tracheal epithelium, and muscles about 120 hours after virus injection (Fig. 1). The Feulgen reaction has shown that similar patches of material are Feulgen-positive, indicating a concentration of DNA in the cytoplasm of infected cells.

[2]Antibiotic suspension was supplied by Dr. D. M. MacLeod of this laboratory.

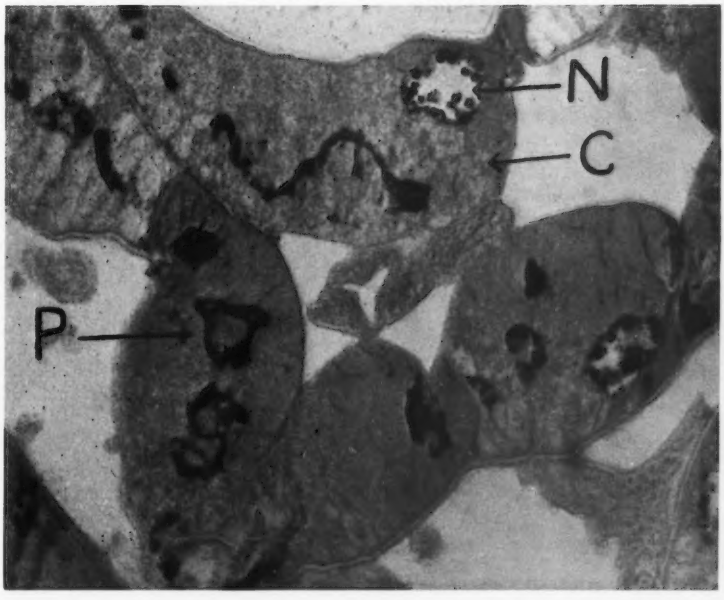
FIG. 1. Section through the silk gland of a *Tipula paludosa* larva infected with *Tipula* iridescent virus showing patches of dark-stained material in the cytoplasm of the cells. N, nucleus; C, cytoplasm; P, patches of dark-stained material. Fixation, Bouin's; stain, Heidenhain's iron haematoxylin. ×750.

FIG. 2. Electron-microscope photograph of a section of a silk gland of *Galleria mellonella* infected with *Tipula* iridescent virus showing groups of virus particles (V), and dense material (D) with which virus particles appear to be associated. N, nucleus; NL, nucleolus, M, mitochondria. ×12,000.

FIG. 3. Electron-microscope photograph of a section through a trachea of *Galleria mellonella* infected with *Tipula* iridescent virus showing virus particles (V), many with clearly defined cores and some that are empty. N, nucleus; NL, nucleolus; T, taenidea of the trachea; V, virus particles. ×20,000.

FIG. 4. Electron microscope photograph of a section of a silk gland of *Galleria mellonella* infected with *Tipula* iridescent virus showing strands of dense material (D), mature virus particles (V), and at L particles with no membranes or poorly defined membranes. ×20,000.

PLATE I

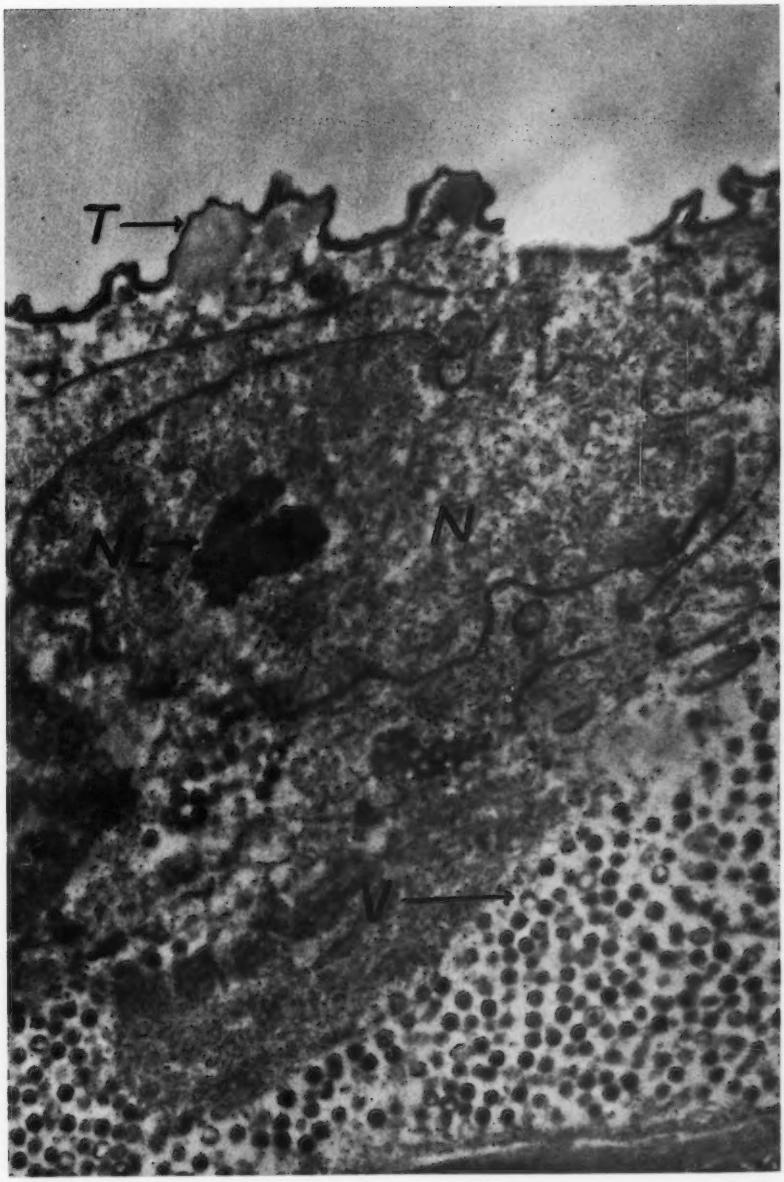


Bird—Can. J. Microbiol.

PLATE II

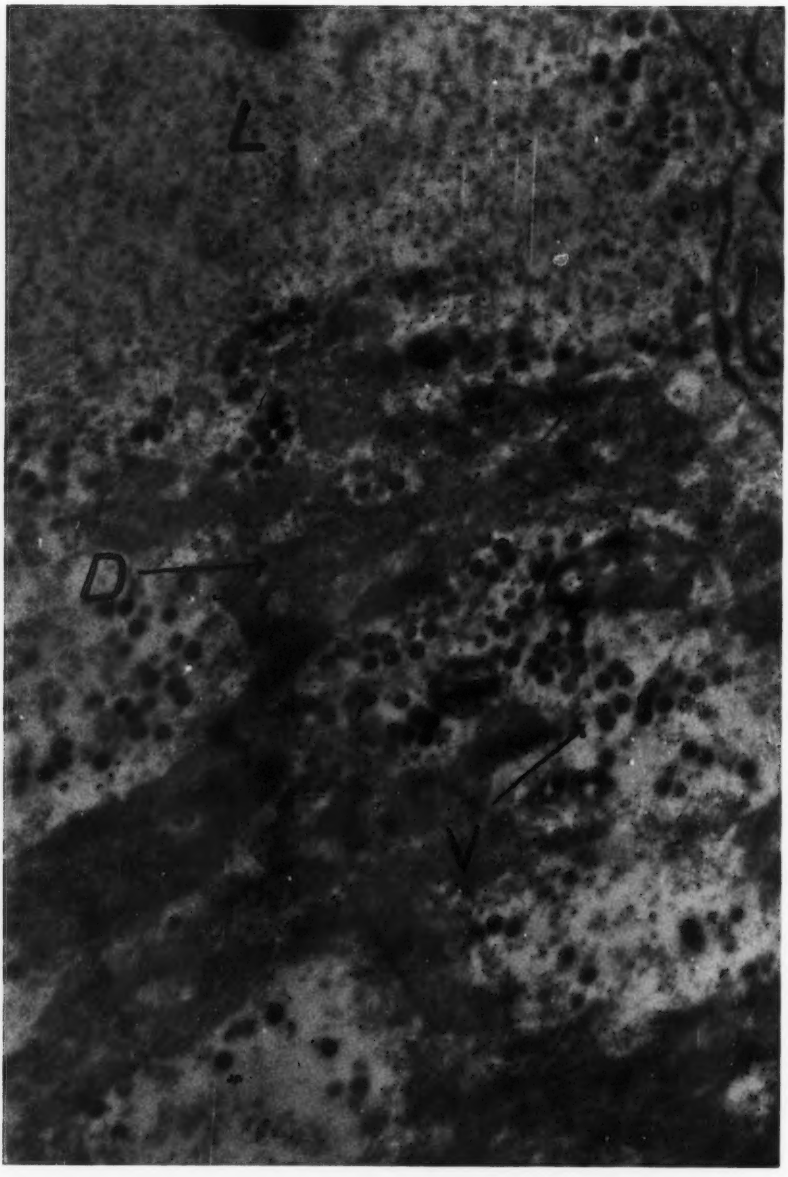
Bird—Can. J. Microbiol.

PLATE III



Bird—Can. J. Microbiol.

PLATE IV



Bird—Can. J. Microbiol.

An electron-microscope photograph of a section through an infected silk gland (Fig. 2) shows several groups of virus particles in the cytoplasm and also dense material in the cytoplasm with which many of the particles appear to be associated. This dense material is probably analogous to that observed in sections under the light microscope which stained with haemotoxylin, and was Feulgen-positive. Figure 3 is a section through an infected tracheal cell. The virus particle is shown to consist of a dark central core surrounded by a membrane. Some of the particles are empty. A study of serial sections has shown that the central part of a particle may be broken off and pulled out during sectioning. This frequently results in a sharp, ragged edge in the remnant of the particle that remains, evident in some of the particles shown in Fig. 3. Figure 4 is a section through the dense material that forms in the cytoplasm of infected cells. It shows virus particles in which the central core is visible, dense particles in which no structure is apparent, and, at the top of the picture, an area where the particles are less clearly defined. The small spheres in this area are possibly the central cores of the virus particles. Close scrutiny reveals that some, if not all, of the particles are surrounded by membranes. "Pockets" of this type of particle are frequently found in heavily infected tissues.

The *Tipula* iridescent virus was consistently infectious and caused complete mortality among *G. mellonella* injected with 7×10^{-6} g to 7×10^{-8} g of virus protein (Table I). Considerable variation in mortality occurred at higher dilutions of the virus, and further tests are necessary to determine the median lethal dosage. Larvae died from 6 to 29 days after injection, the average period to death occurring at about 14 days after injection. Storage in the antibiotic solution for several months at 4° C did not appear to have any effect on the pathogenicity of the virus.

TABLE I

Results of quantitative studies on the injection of *Tipula iridescent* virus into *Galleria mellonella* L.

Date	Quantity of protein* injected (g)	No. of insects	% mortality	Average period to death, in days
10.1.61	7×10^{-6}	19	100	14.4
10.1.61	7×10^{-7}	21	100	12.4
19.1.61	7×10^{-6}	62	100	13.5
31.1.61	7×10^{-7}	26	100	15.8
31.1.61	7×10^{-8}	29	100	15.6
31.1.61	7×10^{-9}	29	96.6	14.9
31.1.61	7×10^{-10}	30	53.3	14.8
31.1.61	7×10^{-11}	30	76.6	16.0
31.1.61	7×10^{-12}	30	70.0	15.8
6.3.61	7×10^{-12}	25	52.0	10.8
6.3.61	7×10^{-13}	28	35.7	9.0

*Protein determinations were made by Dr. P. F. Faulkner of this laboratory.

Discussion

The development of *Tipula* iridescent virus particles in the cytoplasm of certain cells (cells of the silk gland, epidermis, tracheal epithelium, fat body, and muscles) appears to be associated with the appearance of strands of dense

material rich in DNA. In this respect it is similar to the development of nuclear polyhedrosis viruses of insects (2, 3). Strands of dense material and virus particles which appear to be associated with them have also been observed in the cytoplasm of cells infected with a granulosis virus (1).

The virus particle consists of a central core surrounded by a membrane. Many of the particles appear very dense and reveal no internal structure. This may be due to the accumulation of protein around the particles similar to that which occurs during the formation of polyhedrosis and granulosis virus inclusion bodies (1, 2, 3). (The resistance of the virus particles to aging suggests that they are covered by some protective coating.) Some of the particles appear empty. These, apparently, are artifacts produced during sectioning. These and other aspects of the structure of the *Tipula* iridescent virus particle will be studied at much higher magnifications. Particular attention will be devoted to the study of the groups of particles with poorly defined membranes.

Acknowledgments

Grateful acknowledgment is extended to Mr. Roger Laughlin, Unit of Insect Physiology, University of Durham, England, who supplied us with larvae of *Tipula paludosa* Meig., to Dr. K. Smith, Virus Research Unit, Cambridge, England, who supplied us with the *Tipula* iridescent virus, and to Mr. J. M. Burk and Mrs. E. L. Bertolo for technical assistance.

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NOTES

A SIMPLE TECHNIQUE FOR FREEZE-DRYING ANAEROBES

MARCIA D. LUPTON, DAVID W. WHITLOCK, AND GORDON P. LINDBLOM

Many aerobic bacteria, fungi, and yeasts have been preserved successfully by freeze-drying according to the general method of Flosdorf and Mudd (1). Obligately anaerobic bacteria have presented a more difficult problem. Haynes *et al.* (2), at the Northern Utilization Research Branch, USDA, Peoria, Illinois, have had success with aerobic bacteria, fungi, yeasts, and actinomycetes, but stated that attempts to lyophilize anaerobes had been unsuccessful. Recently (Haynes, personal communication) we learned that they now are freeze-drying certain clostridia routinely. Their method consists of centrifuging the cells from a liver broth, resuspending them in bovine serum, and freeze-drying in about 0.1 ml amounts.

While others may have developed satisfactory methods for handling anaerobes in their laboratories, the lack of published literature prompts this report of a method which has given good results in our hands. The procedure has been used for preserving a collection of about 200 anaerobes isolated from several marine environments. The growth medium is used as the suspending fluid, eliminating the use of a centrifuge or special additives such as serum or skim milk.

In our laboratory anaerobic cultures are routinely made in 12-ml vaccine vials containing 10 ml Difco Brewer Thioglycollate Medium, with methylene blue redox indicator. The vials are closed with rubber stoppers and aluminum ring seals, and sterilized in the autoclave. When properly prepared the medium in the vials shows no color change, even after prolonged storage. Since most of our cultures are marine forms, the medium is made in sea water of the required salinity, the pH adjusted, and any precipitate removed by filtration.

In preparation for freeze-drying, 0.5–1.0 ml of a stock culture in this medium is transferred with a syringe to fresh media. A slight negative pressure is induced by evacuating the vial with the same syringe used for inoculation. Heavy growth is apparent after incubation at the proper temperature for 18–24 hours, after which time freeze-drying is carried out.

Bulb-type lyophile tubes of 3-ml capacity are used. In order to displace air, about 1 ml of liquid nitrogen is poured from a sterile beaker into the sterile tubes. The nitrogen vaporizes rapidly, creating an essentially oxygen-free atmosphere. One milliliter of the culture is quickly transferred, with a syringe, to the air-free tube. This is immediately shell-frozen in an alcohol–dry ice slurry and placed on the manifold of the freeze-drying apparatus. Use of a medium containing a methylene blue indicator makes possible easy detection of the presence of oxygen so, if anaerobic conditions have not been maintained during the procedure, the tube may be discarded and a new one prepared. We allow our cultures to dry about 20 hours, after which the tubes are sealed off while under vacuum.

Most methods of freeze-drying require centrifugation of cells followed by resuspension in serum or milk. These time-consuming steps are unnecessary in the method described and the danger of contamination is also minimized.

After 2 years we have yet to encounter a culture of a strict anaerobe, preserved in this manner, which has failed to grow on reconstitution.

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FUSION OF GRANULES OF MYCOBACTERIA AND ITS POSSIBLE GENETIC SIGNIFICANCE

STEPHEN E. JUHASZ

We have shown on serial phase contrast micrographs taken from block preparations of living organisms of *Mycobacterium tuberculosis* var. *bovis* (strain BCG) that granules can fuse to attain larger globular forms (S. E. Juhasz, H. E. Burke, and E. Mankiewicz: Exhibit at the Annual Meeting of the National Tuberculosis Association, Chicago, 1959). These granules probably represent bacterial nuclei. Figure 1 shows the fusion of granules of "filtrable size" (0.2 to 0.4 μ in diameter) into globules over 1 μ in diameter within the club-shaped end of filaments. It was also shown in serial micrographs that larger globules can sprout into typical rods proving once more the richness in nuclear material of granules and globules.

Like the observed fusion of bacterial nuclei in enterobacteria treated with chloramphenicol (1), we found that the fusion of "nuclei" of *Mycobacterium phlei* could be enhanced by the addition of low concentrations of streptomycin to the medium. Fusion of "nuclei" within the same rod was thus observed, but perhaps of greater significance was fusion of "nuclei" from different organisms. Figure 2 shows two bacteria placed at an angle. The upper one contains three granules, as can be seen on B and C. D shows (*a*) the parallel arrangement of the two rods instead of their initial angular disposition, (*b*) the disappearance of the central granule in the upper rod, and (*c*) the simultaneous swelling of the converging rod. On E and F a kind of fusion makes its appearance in the course of which the cell boundaries of the upper rod begin to disappear while the lower shows an increase in both material and volume. The purpose of this investigation was to shed some light on the cytological basis of conjugation.

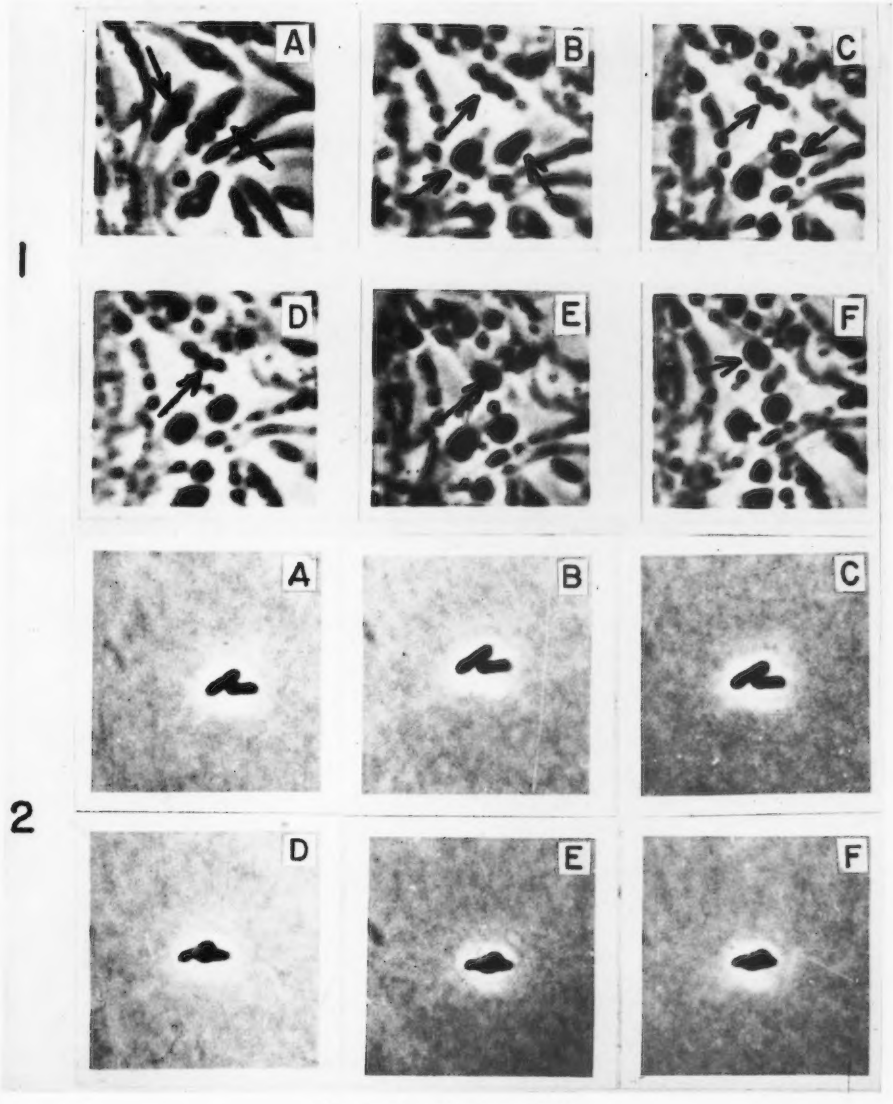

FIG. 1. Magnification ×3200.
FIG. 2. Magnification ×2800.

This study was supported by a National Research Council of Canada grant and the Harrison–Watson scholarship of McGill University, Montreal.

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STUDIES OF CHLOROACETYL DERIVATIVES AS BACTERIAL ANTAGONISTS

W. A. ZYGMUNT

On the basis of the recent report by Levi *et al.* (3) that the sodium salt of N-dichloroacetyl-DL-serine inhibited the development of sarcoma-37 in mice and caused complete regression of the tumor, it was of interest to determine whether D-, L-, and DL-N-dichloroacetylserine and the chloroacetyl derivatives of other amino acids are effective amino acid antagonists in bacteria.

The compounds tested were: chloroacetyl-DL-alanine, chloroacetyl-DL-isoleucine, chloroacetyl-L-leucine, chloroacetyl-DL-phenylalanine, chloroacetyl-L-tryptophan, chloroacetyl-L-tyrosine, chloroacetyl-DL-valine, N-dichloroacetyl-D-serine, N-dichloroacetyl-L-serine, and N-dichloroacetyl-DL-serine (sodium). All of the chloroacetyl derivatives tested were purchased from Nutritional Biochemicals Corporation.

These compounds were tested for their ability to inhibit growth of *Escherichia coli* W in the minimal medium of Davis and Mingioli (2). In addition, the N-dichloroacetylserine compounds were tested in the amino acid basal medium of Barton-Wright (1) using *Leuconostoc mesenteroides* P60, a serine-dependent organism. Neutralized solutions of test compounds, amino acids, and basal media were sterilized by Seitz filtration and portions added to previously autoclaved culture tubes containing graded levels of sterile, distilled water. Only freshly prepared solutions of the test compounds were used. Final assay volumes were 10 ml per culture tube. In the preparation of the inocula, precautions were taken to minimize the carry-over of preformed amino acids by appropriate washing of the culture and by the use of small inocula. Incubation temperatures of 32° and 35° C were used with *E. coli* and *L. mesenteroides*, respectively. Initially, growth was measured turbidimetrically with a Coleman Junior spectrophotometer at a wavelength of 620 mμ. In cases where significant inhibition of bacterial growth had occurred, greater than 20%, viable plate counts were also made using tryptone glucose extract agar (Difco). Since the results of measuring bacterial growth inhibition were closely parallel by the two methods, it appeared that a blockage of true

protoplasmic growth had occurred in those instances where inhibition of bacterial growth was noted.

With *E. coli*, N-dichloroacetyl-DL-serine was the only chloroacetyl derivative showing any inhibition of growth at a level of 1 mg per ml. An increase in the concentration of this test compound to 2 mg per ml increased the level of inhibition only from 5 to 17 per cent. At levels of 2 mg per ml, chloroacetyl-DL-isoleucine and chloroacetyl-DL-valine inhibited the growth of *E. coli* by 39 and 17 per cent, respectively.

In order to determine whether the chloroacetyl derivatives could interfere with the uptake into the cell of their analogous amino acids, the amino acid derivatives were also tested in media containing the respective, individual amino acids as sole nitrogen sources. It was found, however, that single amino acids supported levels of growth markedly lower than those observed in an amino-acid-free basal medium containing simply $(NH_4)_2SO_4$. In the case of the former medium at levels of 0.5, 1.0, and 2.0 mg per ml, DL-isoleucine, DL-valine, DL-leucine, DL-phenylalanine, and L-tryptophan resulted in optical density readings of less than 0.050 after 70 hours of incubation. Although DL-alanine and DL-serine supported growth, the level of growth was approximately one-half of that found with the minimal medium and was subject to a prolonged lag phase. This lag phase was minimized by a process of adaptation. Nonetheless, *E. coli* adapted to grow readily on DL-alanine or DL-serine containing media and was not inhibited by levels of N-dichloroacetyl-DL-serine or chloroacetyl-DL-alanine up to 2 mg per ml.

All of the chloroacetyl derivatives tested at levels up to 2 mg per ml when serving as the sole nitrogen source in the medium failed to support the growth of *E. coli*. This points to the probable extracellular absence, on a constitutive enzyme basis, of the necessary deacylase(s) to form the parent amino acids.

D-, L-, and DL-N-dichloroacetylserine in the presence of 20 μg of DL-serine per ml required for maximal growth of *L. mesenteroides* had no inhibitory effect at levels up to 1.6 mg per ml.

All of the experiments clearly indicate that the chloroacetyl derivatives of the amino acids tested are not potent growth inhibitors. Evidently, with *E. coli* both the synthesis and utilization of the amino acids tested were not readily inhibited by their analogous chloroacetyl derivatives.

Cell permeability studies have not been conducted as yet and, consequently, this phenomenon is not ruled out as the mechanism whereby these compounds are inactive. The data of Meister (4), however, in which it was found that cell-free extracts of *Lactobacillus arabinosus* (classified as *Lactobacillus plantarum* in the 7th edition of Bergey's Manual of Determinative Bacteriology) can in fact transaminate and, presumably, inactive N-chloroacetyllysine and N-chloroacetylornithine, appear to be in agreement with the lack of activity of chloroacetyl compounds as effective amino acid antagonists in bacteria.

No information on previous studies of chloroacetyl compounds as possible amino acid antagonists in bacteria is available. Assuming that amino acid metabolic pathways in tumor cells and bacteria are similar, the antitumor activity reported for N-dichloroacetyl-DL-serine (3) appears to be related to a pathway other than amino acid antagonism.

It is a pleasure to acknowledge the advice and encouragement of Dr. Herbert P. Sarett in this work.

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TECHNIQUES FOR THE STANDARDIZATION OF FLUORESCENT ANTIBODIES USED IN DIAGNOSTIC MICROBIOLOGY

S. SONEA AND J. DE REPENTIGNY

There is a need to set up criteria for defining fluorescent sera suitable for diagnostic use (1, 2). In the present work we suggest the application of techniques which may be useful for this purpose.

To begin with, the total fluorescent intensities of fluorescent sera have been measured in capillaries and related to the fluorescent intensities of known quantities of fluorochromes in aqueous solutions. The microfluorometric method used has already been described (4, 5). Table I shows average intensities of 11 readings, obtained with three horse antisera and with ovalbumin (the latter thrice crystallized), all coupled with fluorescein or with rhodamine B isothiocyanates (3, 6). The fluorescent intensity of 0.73 mg protein/ml solution of fluorescein-coupled tetanus antitoxin is 11,773 units, which corresponds roughly to that of 0.01 mg/ml sodium fluorescein aqueous solution (12,500 units) when examined under the same conditions. This quantity of fluorescein (0.01 mg) is thus probably the amount which has finally been conjugated to 0.73 mg antibody protein. Such measurements have shown that 1 mg of fluorescein is coupled to about 60 mg of protein in our fluorescent sera. Sera coupled with rhodamine B have lower fluorescent intensities. A 10-fold dilution does not necessarily decrease the fluorescent intensities by the same factor, especially when rhodamine B is used. Such measurements could help in eliminating fluorescent sera on which insufficient quantities of fluorochromes have been conjugated.

When tested with ordinary serological methods, no difference in immunological activity was found between the coupled and the uncoupled sera.

Changes in the antibody proteins could be demonstrated when serum samples before and after coupling were compared by the Ouchterlony agar diffusion technique. Figure 2 shows such results obtained with diphtheria antitoxin. After coupling, some secondary lines observed with uncoupled sera were no longer visible, and the main lines were slower to appear. Similar results were obtained with *Staphylococus aureus* antitoxin (Fig. 3).

The capacity of sera to add *specific* fluorescence to microorganisms is not *measured* by the above-mentioned techniques. To fill this gap, the total fluorescent intensity of *bacteria* treated with fluorescent antibodies was compared on an equal-volume basis in smears with the fluorescent intensity of: (1) the same but unstained bacteria, and (2) the same bacteria treated with fluorescent heterologous antibodies or ovalbumin, as controls. Figure 1 summarizes results obtained in two series of experiments. In the first, *S. aureus* (four experiments) and *Gaffkya tetragena* (one experiment) were treated with fluorescent staphylococcal antitoxin. In the second series, *Corynebacterium diphtheriae* (six experiments) and *Corynebacterium pseudodiphtheriticum* (two experiments) were treated with fluorescent diphtheria antitoxin. Their mean fluorescent intensities are represented by a column for each bacterial species.

As seen in Fig. 1 the total fluorescent intensity of *bacteria* treated with fluorescent antibodies can be divided in three distinctive parts. The first one corresponds to the primary fluorescence of bacteria. The second derives

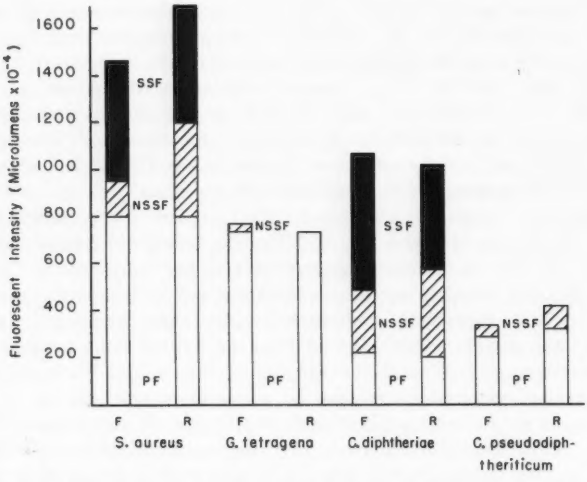

FIG. 1. Comparisons between the intensities of primary fluorescence (PF), nonspecific secondary fluorescence (NSSF), specific secondary fluorescence (SSF), and total fluorescence of bacteria treated with fluorescent antibodies.

F: Fluorescein-isothiocyanate-coupled antitoxins (staphylococcal antitoxin for *S. aureus* and *G. tetragena*; diphtheria antitoxin for *C. diphtheriae* and *C. pseudodiphtheriticum*).

R: same substances coupled with rhodamine B isothiocyanate.

PLATE I

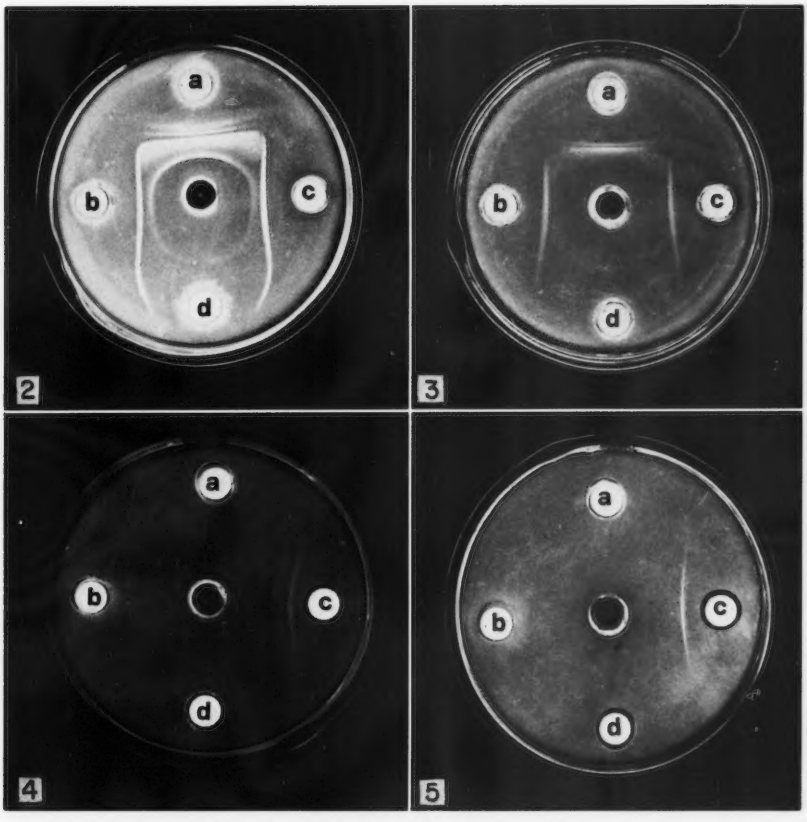

FIG. 2. Antigen (diphtheria toxoid in the central hole) – antibody precipitation in agar: *a*, unlabelled diphtheria antitoxin; *b*, fluorescein-coupled diphtheria antitoxin; *c*, rhodamine-B-coupled diphtheria antitoxin; *d*, unlabelled tetanus antitoxin.

FIG. 3. Same as in Fig 2 but staphylococcus antitoxin was used instead of diphtheria antitoxin. In the central hole staphylococcus toxoid.

FIG. 4. Precipitin agar diffusion test between fluorescein-coupled diphtheria antitoxin and: *a*, sterile culture media; *b*, *S. aureus; c*, *C. diphtheriae; d*, *C. pseudodiphtheriticum.*

FIG. 5. Same as in Fig. 4 but rhodamine-B-coupled diphtheria antitoxin was used instead of the fluorescein-coupled antitoxin.

TABLE I

Microscopic intensities of fluorescent antibodies and fluorochromes (capillary technique)*

Antibodies and controls	Unlabelled		Labelled			
			Rhod. B		Fluores.	
	mg/ml†	Intensity	mg/ml†	Intensity	mg/ml†	Intensity
Diphtheria	6.3	398	5.5	21,863	6.4	>100,000
antitoxin	0.63	—	0.55	4,163	0.64	9,550
Staphylococcus	8.1	386	7.3	15,136	8.2	>100,000
antitoxin	0.81	—	0.73	3,730	0.82	11,000
Tetanus	6.9	427	6.6	18,772	7.3	>100,000
antitoxin	0.69	—	0.66	6,854	0.73	11,773
Ovalbumin	8.8	373	8.5	21,500	8.7	>100,000
	0.88	—	0.85	5,860	0.87	13,363

Fluorochromes	Concentrations			
	1 mg/ml	0.1 mg/ml	0.01 mg/ml	0.001 mg/ml
Fluorescein sodium	>100,000	>100,000	12,500	821
Rhodamine B	89,727	36,772	5,200	722

*One unit being 0.0001 microlumen.
†Mg protein/ml.

from the *nonspecific* "staining" with any fluorochrome-coupled protein; in our experience, coupled ovalbumin gives practically the same staining as heterologous-coupled antisera. The third is due to *specific staining*, which is represented by the difference between the total intensity of the specifically "stained" bacteria and the intensity of *non* specifically stained bacteria. In our experience it ranged from 1/3 to 1/2 of the total intensity. These specific staining intensities are high enough to allow for their differentiation. With high-titered sera, the same technique could be used to determine the practical end point of dilutions adequate for specific fluorochroming. The ability of the fluorescent sera to differentiate between bacterial species was also checked by the agar diffusion technique. Lines were obtained only with the homologous bacteria (Figs. 4 and 5). The intensity of the specific "staining" and the presence of specific lines in agar are objective criteria for any fluorescent serum suitable for diagnostic purposes.

It is suggested that the use of the technique discussed in this report would constitute a real advance toward the standardization of fluorescent sera to be employed in the diagnosis of infectious diseases.

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SENSITIVITY OF SCHIZOPHYLLUM COMMUNE TO CHEMICAL TOXICANTS[1]

YAIR PARAG[2]

An extensive survey has been made for chemicals toxic to *Schizophyllum commune* to be used in the development of a selective technique for the detection of somatic recombination to be based on homozygosity of recessive mutation for resistance (7, 9). Twenty-eight antibiotics, 11 antimetabolites, and 2 organic fungicides were tested for effectiveness against the fungus; most of the antibiotics were selected from the more prominent antifungal agents listed in Spector (10). A qualitative test was made for each agent either by placing a small piece of filter paper saturated with a 1 mg/ml solution of the toxicant or by placing the dry toxicant directly upon the surface of an agar plate inoculated with the macerated mycelium of a standard strain (No. 699), and this was followed by the scoring of the resulting zone of inhibition. Nineteen antibiotics, one antimetabolite, and both fungicides were found to be toxic and were subjected to a further quantitative test (Table I). In this, a known number of mycelial fragments was introduced by pour-plating into agar media containing graded concentrations of each toxicant. Detailed survival curves were established for four toxicants (Fig. 1); the others were only tested in two or three concentrations. Clear-cut *critical concentrations*—i.e., the lowest concentration that permits no visible growth of the test-strain within the 4- to 6-day period required for the appearance of discernible mycelia in control plates—were established for 14 toxicants (Table I). Toxicants that gave survival curves of moderate slope, as well as those that had a graduated effect on the size of the colonies or affected mycelial form rather than the number of survivors, were ruled out as unpromising for use as selective agents.

Several spontaneous resistant mutants appeared upon the test plates containing the antibiotic *polymyxin-B*. Two of these spontaneous mutants were

[1]Work presented to the Department of Biology, Harvard University, as a portion of a dissertation in partial fulfillment of the requirements of the degree of Ph.D.; supported in part by a grant from the National Institutes of Health, U.S. Public Health Service (C-2221), to Professor John R. Raper.
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TABLE I

Activity of toxicants against *S. commune*

Chemicals*†	Critical concn., mg/l.
A. *Effective*	
Bacilomycin-B (et) [m]; Rimocidin (et) [c]; Amphothericin-B (di/me) [a]	1
Gliotoxin (½ et) [a]; Actidione (w) [C—Upjohn]; Nystatin (et) [d]; Hexachlorophene–"G-11" and Dichlorophene–"G-12" (et) [d]	10
Filipin (di) [e]	<2
Azaserine [h]; Patulin (w) [g]; Polymyxin-B (w) [C—Mann]; Acriflavine (w) [C—NBCo]	100
Ustilagic acid (me) [b]	200
Caerulomycin (½ et) [f]	>10
Viridin (di) [b]	>20
Neomycin sulphate [C—Mann]	>300
Thiolutin (non-soluble) [c]	?
B. *Affecting only the form of the mycelium*	
Trichotecin (w) [b] small colonies at	1.5 and 5
Endomycin (w) [e] small colonies at	1 and 10
Auromycin (me) [C—Lederle] small colonies at	20 and 200
Mycophenolic acid (et) [b] "transparent" mycelium at	1 and 20
C. *Non-effective in the qualitative test*	
Gladiolic acid, Griseofulvin [b]; Candicidin [n]; Comirin [l]; Pimaricin [k]; Streptomycin [C—Pfizer]; Na-azide [C]; Antimycin [C—Wisconsin Alumni Research Foundation]; Bacitracin, Penicillin-G, Desoxypyridoxine–HCl, Picolinic acid, Pyridine-3-sulphonic acid, Benzimidasol, Desthiobiotin, Thiouracil, Pantoyl taurine, Sulphanilamide, Sulphathiasole [C—NBCO]	

*The symbols in parentheses represent the solvents of the stock solutions: di = dioxane; et = ethanol, 95%; ½ et = ethanol, 50%; me = methanol; di/me = dioxane and methanol, 1:1; w = water.

†The symbols in brackets represent the sources for the chemicals. C means commercially available through the companies the names of which follow. The others have been very kindly supplied by the following scientists or research laboratories and companies: a = Dr. J. D. Dutcher, The Squibb Institute; b = Dr. P. W. Brian, Imperial Chemical Industries, Akers Research Laboratories; c = Pfizer & Company; d = Sindar Corporation; e = The Upjohn Company; f = Dr. R. H. Haskins, Prairie Regional Laboratories, Saskatoon, Sask.; g = Mann Research Laboratories; h = Dr. C. C. Stock, Sloan-Kettering Institute for Cancer Research, Walker Laboratories; k = Dr. H. N. Porter, Lederle Laboratories; l = Dr. B. K. Kelly, Antibiotic Research Station, Clevedon, Somerset, England; m = Dr. G. H. Warren, Wyeth Institute for Medical Research; n = Merck, Sharp & Dome Research Laboratories.

tested along with eight resistant mutants of independent origin that were isolated after irradiation with ultraviolet light by Mr. A. S. Flexer. In a typical test, a mutant strain, PR1, $A^{41}B^{41}$ *pol*^{r}*-1* and derived directly from No. 699, was crossed with a wild strain, No. E702, $A^{47}B^{47}$ *pol*^{s}*-1* and isogenic to No. 699; the trait for resistance segregated 1:1 with its wild allele. From this cross, strain PR1-2, $A^{47}B^{47}$ *pol*^{r}*-1* and also isogenic to No. 699, was isolated, and this was used in the subsequent tests. Dikaryons established by crosses between these isogenic strains and homozygous for *pol*^{r}*-1*, homozygous for *pol*^{s}*-1*, and heterozygous for the two alleles were macerated and tested on a graded series of concentrations of polymyxin. *Pol*^{r}*-1* was found to be recessive—though not completely—to its wild allele (Figs. 2 and 3). The other nine mutations were found, by complementation tests in dikaryons, to be allelic to *pol*^{r}*-1* and, by tests in dikaryons with wild type, they were found also to be recessive.

These tests led to the further observation that the exposure of the heterozygous dikaryon to an inhibiting concentration of polymyxin always resulted in the establishment of a resistant homokaryon. Platings of macerates of the

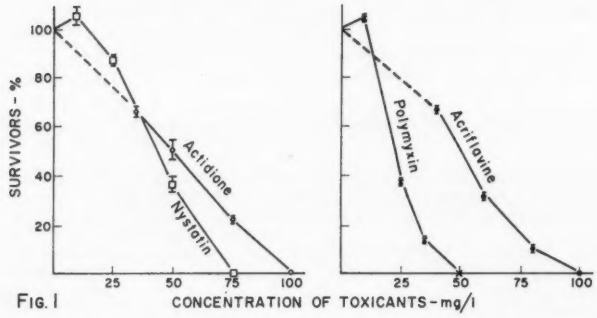

FIG. 1

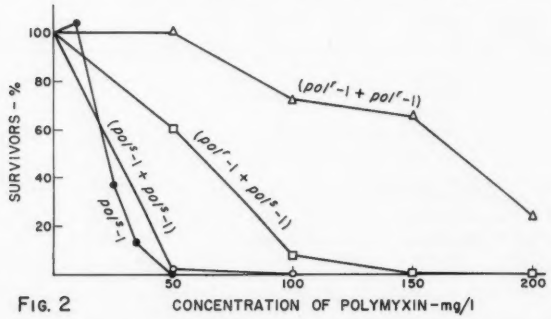

FIG. 2

FIG. 1. Dosage–response curves for the action of four toxicants upon the homokaryon of *S. commune*.

FIG. 2. Dosage–response curves for the action of polymyxin-B upon three dikaryons—homozygous resistant, homozygous sensitive, and heterozygous—and a sensitive homokaryon.

heterozygous dikaryon in medium containing 100 mg/l. polymyxin resulted in the growth of many vigorous survivors (8.2%; Figs. 2 and 4). These putative, resistant, dikaryotic mycelia were found, upon isolation, however, to be homokaryons and to have the genetic constitution of the resistant component of the dikaryon, $A^{41}B^{41}$ *pol*^{r}*-1*. Both the typical survival curve as well as the emergence of the resistant homokaryotic component were confirmed in each of a number of genetically different heterozygous dikaryons. It is clear, therefore, that the resistant component homokaryon is regularly selected out of the heterozygous dikaryon upon media containing polymyxin. It is not clear, however, whether this dedikaryotization is induced by the antibiotic, or whether the resistant homokaryon is only selected out following infrequent spontaneous dedikaryotization (2).

Since the purpose of developing these mutant strains was the selection of somatic recombinants, a number of sensitive and resistant homokaryons and dikaryons were tested on the proposed selective media—minimal medium (8) containing appropriate concentrations of polymyxin—to determine the suitability of the various strains for their intended use. Surprisingly, all were found to be sensitive on these media. Further experiments were performed upon media containing the different components of the selective and non-selective

PLATE I

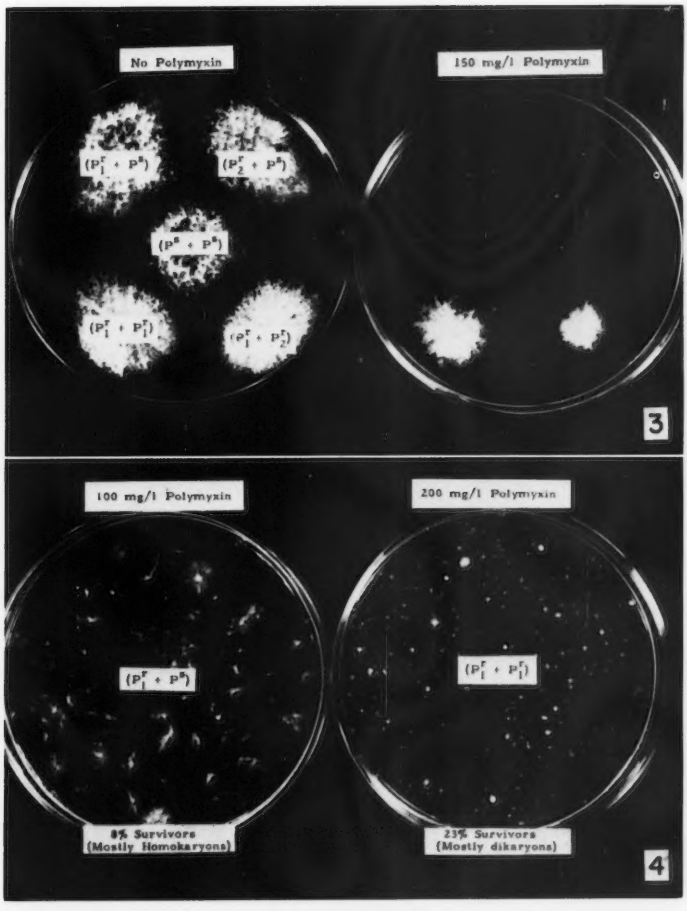

FIG. 3. Growth response to polymyxin of five dikaryons—one homozygous sensitive, one homozygous resistant, two heterozygous, and one heterozygous in repulsion for two independent, apparently allelic, mutations for resistance. Left: no polymyxin. Right: polymyxin, 150 mg/l.

FIG. 4. Comparison of number and size of colonies originating from macerates of a heterozygous dikaryon (left) and a homozygous resistant dikaryon on 100 mg/l. and 200 mg/l. polymyxin, respectively.

media—mineral salts, dextrose, peptone, asparagine, Bacto agar, Noble's agar, and polymyxin—in many different combinations. These tests revealed that the genotypically resistant mycelia were sensitive or resistant with Noble's agar or Bacto agar, respectively. This may be because either (*a*) Noble's agar lacks some component, present in Bacto agar, that is needed for the development of resistance by *pol*^{r}*-1*, or (*b*) Noble's agar has some component, absent in Bacto agar, that increases the toxicity of polymyxin. It is difficult to explain the phenomenon on the basis of the mechanisms of action of polymyxin that have been suggested for bacteria (3) and for green algae (1).

The results of this study have practical applications in investigations of several kinds, some of which have already been initiated (4, 5, 6): (1) critical concentrations of 14 toxicants that are effective against *Schizophyllum commune* have been determined, and these agents are now ready for use in the screening of resistant mutations; (2) the restriction of growth caused by some antibiotics can be generally useful in various genetic studies, such as segregation tests and mutant-hunts (for example, endomycin, in a concentration of 1 mg/l., proved to be very effective); (3) the relationship between a single-gene mutation for resistance against polymyxin and an analyzable nutritional condition affecting resistance can possibly serve as a tool in the investigation of the mode of action of the drug; (4) the breakdown of the heterozygous resistant dikaryon can be used for the selection, as a sectored homokaryon, of a known dikaryotic component; (5) polymyxin-resistant mutations are available for the study of somatic recombination, and the properties of the medium for such a study have been determined.

The author wishes to express his sincere appreciation to Dr. A. H. Ellingboe and Mr. A. S. Flexer for certain of the strains used in this study and to the numerous individuals and institutions listed in Table I for the samples of antibiotics that they so generously provided; and above all the author wishes to thank Professor John R. Raper for his encouragement, advice, and invaluable suggestions, without which this work could not have been accomplished.

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SOME MEDIA USED IN AN ATTEMPT TO ISOLATE AND CULTURE THE MYCETOMAL MICROORGANISMS OF SITOPHILUS WEEVILS

A. J. MUSGRAVE[1] AND L. A. MCDERMOTT[2]

Various microorganisms seem to live in different species of insects in an association of mutual benefit. Many attempts have been made to isolate such microorganisms and culture them *in vitro* and several authors have claimed success (2, 4, 5, 8). One of the problems is that of obtaining satisfactory evidence that a microorganism isolated from an insect and cultivated *in vitro* is the same organism as that seen in a particular location inside the insect host (4). Some investigators (1, 9) have been able to surmount this difficulty by means of reinfection experiments. Insects artificially rendered free of microorganisms suffered in growth and development but recovered when the organisms, cultured *in vitro*, were reintroduced. Such experiments are most easily done when the infection is acquired orally.

In the *Sitophilus* weevils the mutualistic microorganisms are harbored in larval mycetomes and in adult mesentera and female reproductive organs of the insects and are inherited congenitally through the female weevil by an internal infection of the oocyte (6). There is evidence that there is no oral infection. The mycetomal microorganisms of *Sitophilus* weevils have not so far been successfully cultured *in vitro*. Other microorganisms of the microflora have, however, been isolated and identified (3).

This note describes briefly the technique and media used in an unsuccessful attempt to isolate the mycetomal microorganisms of *Sitophilus* weevils.

While there are several methods of artificially freeing *Sitophilus* weevils of their mycetomal microorganisms (6, 7), there are, as yet, no proven methods of reinfection. Therefore, in the work described here, an attempt was made to "tag" the mycetomal microorganisms by keeping them under frequent close observation so that any change of form or the occurrence of sporulation could be noted and identified as deriving from the original organisms isolated.

Isolations of crushed tissues harboring the mycetomal microorganisms were made in drops of culture media on glass cover slips which were then inverted over plain or welled glass slides. The cover slips were sealed to the slides by vaseline or vaspar. The little cultures set up in this way could be observed by the phase contrast microscope. The usual precautions were taken to achieve sterility of glassware and other materials and checks on this were made from time to time. Insect tissue was sterilized either by ethyl alcohol or by Hyamine (2000 p.p.m.) and by washing in sterile distilled water.

In selecting culture media the speculation (6) that the microorganisms are actinomycetes prompted the use of media listed for these organisms (11). Trehalose was used in some media, as there is evidence (10) that this is the predominant insect blood sugar. A vertebrate tissue culture medium (No. 1066 of the Connaught Medical Laboratories, Toronto) was also employed in the hope that growing mycetomes or mesentera might facilitate the growth of the organisms.

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The following 35 media were employed in 126 primary and 24 subsidiary isolations, the number of times each medium was used being given in parenthesis after it: asparagine–mannite agar (2), asparagine–mannite broth (4), asparagine–trehalose broth (3), blood agar (8), Czapek's agar (1), Czapek's broth (3), Czapek's trehalose broth (1), glucose–asparagine agar (1), glucose–asparagine broth (1), glucose–asparagine–trehalose broth (1), glycerin–asparagine agar (2), glycerin–asparagine broth (1), macerated larvae medium (1), microinoculum agar (4), microinoculum broth (2), miscellaneous media incorporating insect tissue (8), nutrient beef extract agar (1), nutrient glucose broth (2), nutrient glucose – trehalose broth (1), nutrient salt agar (1), nutrient salt – trehalose broth (1), Richard's agar (2), roach blood (2), soil extract agar (4), soil extract broth (8), yeast–glucose agar (2), yeast–glucose broth (1), yeast–glucose–trehalose broth (1), yeast–trehalose broth (5), yeast–peptone agar (2), yeast–peptone broth (9), yeast–peptone–trehalose broth (35), 1066 agar (8), 1066 broth (21), 1066 trehalose broth (1).

Cocci were observed in about one-third of the isolations, small rods in a few. In addition, in some of the isolations the mycetomal microorganisms could be discerned for a month or more in "lifelike" condition: various subcultures from some of these were either unsuccessful or eventually yielded a preponderance of cocci or small rods (identified as *Bacillus cereus* group), regarded as part of the normal regular flora—a notion that subsequently received some support (3).

There was no satisfactory evidence that the mycetomal microorganisms had been cultured.

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ANAEROBIC DISSIMILATION OF GLUCOSE BY VIBRIO COMMA[1]

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Glucose degradation by *Vibrio comma* has been postulated by Krishna Murti and Shrivastava (3) to proceed via a pathway involving phosphorylated intermediates. These investigators have demonstrated the presence in crude cell-free extracts of enzymes of the glycolytic pathway, as well as several enzymes operative in the hexose monophosphate pathway.

To provide a more definitive answer as to the relative importance of these pathways in intact resting cells, we inquired into (1) the quantitative nature of the fermentation, and (2) the fate of labelled substrate.

Vibrio comma Inaba, strain 9459 of the American Type Culture Collection, was used in the experiments described below. The culture was maintained by monthly transfer in broth containing 1% tryptone and 0.5% yeast extract at pH 7.5. The stock culture was grown aerobically at 30° C for 20 hours. Refrigeration resulted in extensive lysis and subsequent death. This difficulty was obviated by storage at room temperature.

To obtain cells for the metabolic and isotope experiments, a transfer (0.5% inoculum) from stock broth was made to a 500-ml flask containing 100 ml of fresh tryptone – yeast extract broth. The flask was shaken for 15 hours at 30° C and then transferred to a second flask of tryptone – yeast extract broth. The cells were harvested after 11–12 hours' growth at 30° C, washed twice with 30 ml 0.85% saline solution, and suspended in saline solution. Cell concentrations were determined from a previously prepared standard curve relating dry weight to optical density at 660 mμ. Freshly harvested and washed cell suspensions were used in all experiments.

The products of glucose fermentation at pH 7.0 by resting cell suspensions of *V. comma* are shown in Table I.

TABLE I

Products (μmoles) of glucose fermentation by resting cell suspensions of *V. comma*

Glucose used	13.95
Products formed	
Ethanol	7.07
Acetic acid	5.46
Formic acid	10.18
Lactic acid	7.40
Succinic acid	3.86
Glycerol	0.15
2,3-Butanediol	0.17
Diacetyl and acetoin	Trace
Carbon recovery, %	88.3
Oxidation–reduction balance	0.95

Per flask: 30 μmoles glucose per ml; 130 μmoles sodium bicarbonate per ml; 18 μmoles inorganic phosphate per ml; 30 ml of cell suspension; total volume of 200 ml; pH 7.0; atmosphere 100% CO_2; 30° C.

[1]This investigation was supported in part by research grants from the National Science Foundation (G-4023, G-9849) and the United States Public Health Service (E-1467).

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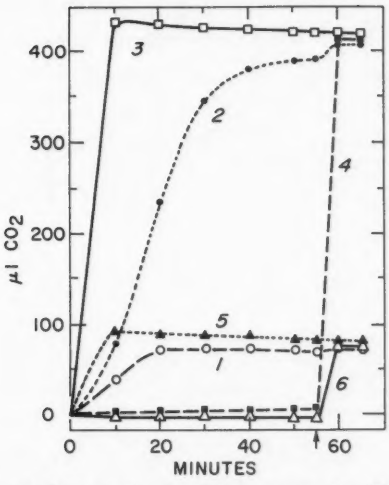

FIG. 1. Rate of glucose fermentation by *Vibrio comma*. Per Warburg cup: 0.5 ml (8 mg dry weight) cells, 6 μmoles inorganic phosphate, total volume 2.1 ml; atmosphere 5% CO_2, 95% N_2; pH 7.0; 30° C. When present: 20 μmoles glucose, 20 μmoles sodium bicarbonate. 1, glucose, sulphuric acid tipped in at 55 minutes; 2, glucose and sodium bicarbonate, sulphuric acid tipped in at 55 minutes; 3, sodium bicarbonate, sulphuric acid tipped in initially; 4, sodium bicarbonate, sulphuric acid tipped in at 55 minutes; 5, no glucose or bicarbonate added, sulphuric acid tipped in initially; 6, no glucose or bicarbonate added, sulphuric acid tipped in at 55 minutes.

Hirsch (2) and Linton *et al.* (4) observed the formation of small amounts of CO_2 during the anaerobic degradation of glucose in complex media by growing cultures of *V. comma*. Since no CO_2 was detected in the present experiments, it seemed advisable to investigate further the absence of gas formation during the fermentation of glucose by non-proliferating cells. Data illustrating acid production at pH 7.0, measured manometrically as CO_2 released from bicarbonate buffer, are shown in Fig. 1. The sodium bicarbonate was recovered quantitatively from all flasks as non-metabolic CO_2 released either by metabolic acids produced during fermentation or chemically by addition of H_2SO_4. Similar experiments performed in various hydrogen ion concentrations, buffers, and anaerobic atmospheric environments yielded essentially the same results. It was concluded that glucose fermentation by *V. comma* yields acid but no gas under the conditions employed.

The large amounts of ethanol, acetate, and formate, and the absence of CO_2 formation, suggest that the Embden–Meyerhof glycolytic scheme functions, at least in part, as the mechanism whereby glucose is degraded anaerobically by *V. comma*.

The products of glucose fermentation by *V. comma* are similar to those of other enteric pathogens. *Vibrio*, *Salmonella*, and some strains of *Shigella* are heterofermentative, non-aerogenic organisms which produce large amounts of acids from glucose. They differ from *E. coli* in their inability to ferment lactose and to produce gas from formate during the anaerobic degradation of glucose.

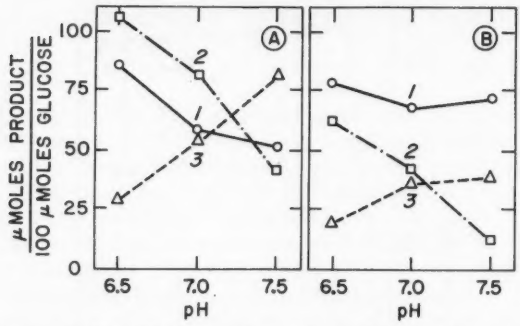

FIG. 2. Influence of pH and buffer on the products of glucose fermentation by *Vibrio comma*. Per Warburg cup: 0.5 ml cells (10 mg dry weight); 20 μmoles glucose per ml; bicarbonate buffer: pH 6.5—41 μmoles per ml, pH 7.0—129 μmoles per ml, pH 7.5—410 μmoles per ml; total volume 3.0 ml; atmosphere 100% CO_2, 30° C. A, no phosphate; B, 20 μmoles phosphate per ml; 1, ethanol; 2, acetic acid; 3, lactic acid.

The influence of environmental conditions on the relative yields of some of the major products of glucose fermentation is shown in Fig. 2. The molar ratios of these products were observed to be markedly dependent upon the pH and on the nature of the buffer used. As may be noted, using bicarbonate buffer (A), the yield of ethanol and acetate decreased while that of lactic acid increased with increasing pH. When the fermentation was carried out in bicarbonate buffer containing phosphate (B), the amount of ethanol remained constant, while that of acetate decreased and of lactate increased with increasing alkalinity. It is apparent that the fate of pyruvate in *V. comma* is dependent upon the hydrogen ion concentration. In the presence of phosphate, the effect of pH is opposite to that noted in the homofermentative lactic acid bacteria (1) and in *E. coli* (5).

If the Embden–Meyerhof glycolytic scheme operates in *V. comma* as a mechanism for the anaerobic dissimilation of glucose, it could be expected that compounds known to inhibit certain glycolytic enzymes would inhibit the fermentation. Data illustrating the influence of sodium fluoride and iodoacetate on glucose fermentation are shown in Table II. The marked inhibitory activity of these compounds is taken as presumptive evidence for the presence of the Embden–Meyerhof scheme.

TABLE II

Effect of inhibitors on fermentation of glucose

Inhibitor	Q_{CO_2} (μl/mg dry weight/hr)		% Inhibition
	Control	Exptl.	
NaF (1×10^{-2} *M*)	27.0	7.0	74.1
IAA (2×10^{-3} *M*)	104.0	1.1	100

NaF (sodium fluoride): 10 mg cells (dry weight), 11 μmoles sodium bicarbonate, 6 μmoles inorganic phosphate, 10 μmoles glucose, 5% CO_2: 95% N_2 atmosphere, pH 7.0, total volume of 2.0 ml. Shaken in Warburg bath at 30° C for 3 hours.

IAA (iodoacetate): 10 mg cells (dry weight), 258 μmoles sodium bicarbonate, 20 μmoles glucose, 100% CO_2 atmosphere, pH 7.0, total volumes of 2.0 ml. Shaken in Warburg bath at 30° C for 3 hours.

TABLE III

Degradation of ethanol and acetate from fermentation of position-labelled glucose by *V. comma*

Substrate	Specific activity (c.p.m./μmole)			
	Ethanol		Acetate	
	CH_3	CH_2OH	CH_3	COOH
Glucose-3,4-C^{14} (362 c.p.m./μmole)	12.5	12	4.3	7.8
Glucose-2-C^{14} (253 c.p.m./μmole)	0.0	121	0.0	122

Per 125-ml Warburg vessel: 3.0 ml resting cell suspension, 38 μmoles glucose/ml, 135 μmoles bicarbonate/ml, water to 30 ml; atmosphere 100% CO_2; pH 7.0; 30° C. No carrier added. Specific activity is expressed in counts per minute per μmole of compound.

Supporting data for the operation of this pathway were obtained from the fermentation of specifically labelled glucose-C^{14} by a resting cell suspension in bicarbonate buffer at pH 7.0, under a 100% CO_2 atmosphere.

The data of Table III indicate that the vibrio fermentation does not follow the Entner–Douderoff pathway. If ethanol and acetate arose by this scheme, C^{14} from positions 3 and 4 of glucose would be found in the methyl carbons of these products. Only traces of the isotope were detected in positions 2 of ethanol and acetate. The absence of labelling in the methyl carbons, together with a lack of production of metabolic CO_2, also rules out the existence of any other known hexose monophosphate pathway.

The fermentation of glucose-2-C^{14} yielded carbinol-labelled ethanol and carboxyl-labelled acetate. Half of the label added in the substrate was contained in the 1 position of ethanol and half in the 1 position of acetate. The triose phosphate derived from the 1, 2, and 3 carbons of glucose may be considered metabolically equivalent to the triose phosphate derived, respectively, from the 6, 5, and 4 carbons.

These data are taken to indicate that, under the conditions provided, the vibrio fermentation follows the classical Embden–Meyerhof glycolytic pathway exclusively. This conclusion is supported by the effective inhibition of the fermentation by sodium fluoride and iodoacetate, by the absence of detectable CO_2 as a metabolic product, and by the nature of the products formed.

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EFFECT OF SIZE OF INOCULA ON THE GROWTH OF SHIGELLA SONNEI IN A CHEMICALLY DEFINED MEDIUM[1]

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Shigella sonnei may be grown readily in the chemically defined medium of Erlandson and Mackey (1). This medium contains inorganic salts, glucose, aspartic acid, thiamine, and niacin. Erlandson and Mackey reported that *Shigella flexneri* grew in this medium with a minimum inoculum of 10^2 cells per ml.

The present study was undertaken to determine the effect of the size of inocula of *S. sonnei* on the growth in this defined medium. Furthermore, the growth of *S. sonnei* in the defined medium was compared with the growth in nutrient broth.

Twenty-four-hour cultures of *S. sonnei* grown in nutrient broth were washed three times in saline solution, and the cell suspensions were adjusted by dilution to obtain the desired cell populations. The size of the inocula was determined by plate-counting using nutrient agar plates. The following inoculum sizes were studied: 10, 100, 1000, 2000, 3000, 4000, 5000, 10,000, 20,000, and 50,000 cells per ml. These values were determined by serial dilutions, which were plated and counted, and also by direct counting in the Petroff–Hauser counting chamber. Each inoculum size was inoculated into 40 tubes of the synthetic medium, incubated at 37° C for 24 and 48 hours, and growth determined in the Coleman Junior spectrophotometer (with wavelength set at 575 mμ). Tubes which had questionable growth were additionally studied; 0.1 ml of the suspension was plated and streaked on nutrient agar plates, incubated, and examined.

TABLE I

The effect of the size of inocula on the growth of *S. sonnei* in a chemically defined medium

Inoculum (cells/ml)	Optical density (at 575 mμ)*			
	24 hr	48 hr	72 hr	96 hr
10	0	0	0	0
100	0	0	0	0
1,000	0.11	0.63	0.94	1.05
2,000	0.23	0.70	0.89	1.10
3,000	0.30	0.77	0.91	1.03
4,000	0.38	0.89	1.10	1.08
5,000	0.40	0.88	1.00	1.15
10,000	0.52	0.79	1.14	1.17
20,000	0.48	0.83	1.31	1.29
50,000	0.53	0.89	1.22	1.19

*Average values obtained from 40 tubes.

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Although growth occurred in nutrient broth with the smallest inoculum, i.e. 10 cells, in the synthetic medium the minimum inoculum to yield growth was 1000 cells. Eighty-two and one-half per cent of the tubes inoculated with 1000 cells showed growth; 95% of the tubes inoculated with 2000 cells resulted in growth; 97.5% of the tubes inoculated with 3000 and 4000 cells produced growth; and 100% of the tubes inoculated with 5000 or more cells produced growth. The amount of growth varied considerably depending upon the size of the inoculum; the data are summarized in Table I. Although the rate of growth from smaller inocula was initially slower, approximately the same total growth was eventually reached with all inocula containing more than 1000 cells. Similar results were observed by Erlandson and Mackey using *S. flexneri* (1).

These results indicate the importance of the size of inocula when studying the nutritional requirements of *S. sonnei* in synthetic media. Determination of the minimal inoculum size which will produce growth in 100% of the tubes is necessary. Furthermore, it appears clear that the culture tubes being observed for growth should be kept for a minimum of 96 hours.

1. Erlandson, A. L., Jr. and Mackey, W. H. Nutrition of *Shigella:* Growth of *Shigella flexneri* in a simple chemically defined medium. J. Bacteriol. **75**, 253–257 (1958).

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